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Protein corona and nanoparticles: how can we investigate on?

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Nanoparticles (NPs) represent one of the most promising tools for drug-targeting and drug-delivery. However, a deeper understanding of the complex dynamics that happen after their *in vivo* administration is required. Particularly, plasma proteins tend to associate to NPs, forming a new surface named the 'protein corona' (PC). This surface is the most exposed as the 'visible side' of NPs and therefore, can have a strong impact on NP biodistribution, targeting efficacy and also toxicity. The PC consists of two poorly delimited layers, known as 'hard corona' (HC) and 'soft corona' (SC), that are affected by the complexity of the environment and the formed protein-surface equilibrium during *in vivo* blood circulation. The HC corona is formed by proteins strongly associated to the NPs, while the SC is an outer layer consisting of loosely bound proteins. Several studies attempted to investigate the HC, which is easier to be isolated, but yielded poor reproducibility, due to varying experimental conditions. As a consequence, full mapping of the HC for different NPs is still lacking. Moreover, the current knowledge on the SC, which may play a major role in the 'first' interaction of NPs once *in vivo*, is very limited, mainly due to the difficulties in preserving it after purification. Therefore, multi-disciplinary approaches leading to the obtainment of a major number of information about the PC and its properties is strongly needed to fully understand its impact and to better support a more safety and conscious application of nanotechnology in medicine. © 2017 Wiley Periodicals, Inc.

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INTRODUCTION

In the last two decades, pharmaceutical research programs have developed a progressively growing interest in nanomedicines for diagnostics, therapeutics and specific drug-delivery¹ as confirmed by an increasing number of nanomedicines fully on market. In order to speed up the translatability of nanomedicines, understanding their fate *in vivo* is pivotal.

In vivo, nanomedicines are immediately covered by proteins from the bloodstream leading to the formation of what is called the 'protein corona' (PC).^{2,3} When the PC forms on NPs, it could govern the fate and

successes/failures of nanomedicines in terms of efficacy, targeting, toxicity, cellular interaction, cellular uptake, and biodistribution.^{4–8} Protein composition, architecture and structure are normally characterized by well-known protocols that have been applied to the PC. The evidence is that to-date PC (or better 'protein corona + nanomedicine') is poorly characterized in terms of chemico-physical and structural features. Therefore, in this review, we aim to comment on the most relevant possibilities in terms of experimental methodologies to more completely characterize these new entities, and to furnish useful data to better predict the fate and efficiency of these drug delivery systems *in vivo*.

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HARD AND SOFT CORONA

The PC is frequently described as being composed by a 'hard' (HC) and a 'soft' (SC) portions, with the

1 binding force of the proteins to nanomaterial often
2 considered as the discriminating criterion.^{9–12} Thus,
3 the HC is generally defined as ‘the corona composed
4 by tightly bound proteins that do not readily desorb
5 from the nanomaterial’, whereas the SC is indicated
6 as ‘the corona featured by loosely bound proteins’.¹³

AQ4 7 In addition to these definitions, Sakulku
8 et al. (2013) separated the total PC of their SPION
9 into three parts: soft, hard and tightly bound, sug-
10 gesting the possibility to further discriminate another
11 level of binding force for protein surrounding the
12 nanomaterial.¹⁴

13 However, the definition of HC and SC can also
14 take into account more complex issues relating to
15 thermodynamic and kinetic matters, the interaction
16 with nanomaterial and the functional/ biological
17 responses.

18 As reported,¹⁵ from a thermodynamic point of
19 view, the HC adsorbs onto the surface of NPs in a ther-
20 modynamically favorable manner with a large net
21 binding energy of adsorption (ΔG_{ads}). This binding
22 energy determines the stability of the protein–
23 nanomaterial complex, as a consequence, proteins that
24 adsorb with a large ΔG_{ads} have a low probability of
25 desorption and tend to stay associated with the nano-
26 material.¹⁶ On the other hand, proteins that adsorb
27 with a small ΔG_{ads} , easily desorb and return to solu-
28 tion, as in the case of SC. Thus, it is possible to divide
29 protein adsorption and desorption into ‘fast’ and ‘slow’
30 components. According to this idea, Cedervall
31 et al. modeled total plasma protein adsorption using a
32 bi-exponential function.¹⁷ The Cedervall’s model
33 implicitly divides protein adsorption and desorption
34 into ‘fast’ and ‘slow’ components, with its own ‘effec-
35 tive’ k_{on} and k_{off} . Since the fast and slow components
36 of adsorption and desorption presumably represent the
37 hard and soft coronas, in some recent papers,^{10,13,18}
38 the SC and HC, respectively, are alternatively indicated
39 with the terms ‘fast component’ and ‘slow component’,
40 referring to desorption processes. On the other hand,
41 considering the adsorption process, the fast and slow
42 terms must be inverted. Adsorption/ desorption times
43 and kinetic curves are unique to each nanomaterial
44 and depend on many parameters. In this experiment,
45 protein desorption to *N*-isopropylacrylamide/*N*-tert-
46 butylacrylamide (NIPAM/BAM) copolymer nanoparti-
47 cles, showed a mean lifetime of 10 min for the fast
48 component (SC), and 8 h for the slow component
49 (HC). However, it remains almost impossible to clearly
50 establish global standard parameters belonging to fast
51 and slow components or, in other words, to SC
52 and HC.

53 In order to better define the HC and SC,
54 another debatable aspect consists in the interaction

57 with nanomaterial. The HC is frequently considered
58 as the portion of the PC directly interacting with the
59 nanomaterial and the SC as the external portion of
60 the PC, which is interacting with the inner HC via
61 protein–protein interactions. In support of this
62 vision, Simberg and colleagues identified specific pro-
63 tein domains as responsible for HC adsorption on
64 their iron oxide NPs. In particular, the authors attrib-
65 uted ‘domain 5’ (D5) for the adsorption of high
66 molecular weight kininogen onto iron oxide nano-
67 particles.¹⁹ The precise mechanisms involved during
68 adsorption and their relative contributions strongly
69 depend on the proteins which interact and on the
70 physicochemical properties of the nanomaterial; thus,
71 it is very difficult, and not always possible, to deter-
72 mine the protein domain that interacts with the
73 nanomaterial, especially if the NPs are incubated in a
74 complex fluid such as plasma.

75 It is also necessary to consider that, the HC
76 results from both protein/protein and protein/nano-
77 material interactions and that the stability of the PC
78 is strongly dependent on both the type and the bind-
79 ing force of proteins forming the HC, and that this
80 should be known to predict the *in vivo* behaviour.
81 Recently, Lynch et al. demonstrated the importance
82 of the HC on the physiological response to a nano-
83 material.²⁰ In their experiments, the HC remained
84 adsorbed onto the nanomaterial during biophysical
85 events such as endocytosis, and even after transloca-
86 tion to a new physiological environment. On the con-
87 trary, the SC rapidly dissociated during translocation
88 and was quickly lost. Moreover, the HC reflects the
89 journey of the nanomaterial in the body compart-
90 ments. For example, a nanomaterial that enters the
91 blood through the lung may display dramatic differ-
92 ences in HC compositions, and in the resulting physi-
93 ological responses with respect to the same
94 nanomaterial directly injected in the bloodstream.²¹
95 However, this biological/functional distinction
96 between HC and SC is not supported by solid data
97 concerning the SC, but is only limited on speculations
98 based on HC results.

99 Similarly, the dynamics involving the SC equi-
100 librium after *in vivo* administration represent a
101 critical point to define the circulation stability of
102 nanomaterials.

103 Overall, a precise and specific distinction
104 between the HC and SC is hard to be defined due to
105 poor experimental evidences aiming to univocally
106 individuate and unambiguously discriminate the cri-
107 teria. Therefore, multiple characterizations must be
108 utilized to discriminate between the HC and SC and
109 more completely understand the role of the PC on
110 the fate of nanomedicines (Table 1).

TABLE 1 | Schematic Illustration of Hard Corona and Soft Corona Characteristics

Hard Corona	Soft Corona
Tightly bound proteins	Loosely bound proteins
$\uparrow \Delta G_{\text{abs}} $	$\downarrow \Delta G_{\text{abs}} $
$\downarrow k_{\text{off}}$	$\uparrow k_{\text{off}}$
Directly interacting with nanomaterials	Protein–protein interaction (and with nanomaterial too?)
Stable on NP surface and able to influence the functional response	Fleeting on NP surface and irrelevant for the functional response

NP, nanoparticle.

ANALYTICAL METHODS FOR CORONA EVALUATION

The study of the PC can be separated into different points of view: analysis of PC structure (i.e., thickness), protein quantification (quantitative or semi-quantitative approach), study of protein affinity and stoichiometry, evaluation of protein conformation, analysis of NP–protein interaction and identification of the PC composition (qualitative approach). Overall, on the basis of the analytical methods applied in a study, two different approaches of investigation could be identified: *in situ* or *ex situ*.¹⁵ *In situ* techniques measure the NPs–PC complex directly into the protein solution where NPs are dispersed. Following this approach, the excessive sample manipulation is avoided and the incubation context is preserved allowing a reliable measurement of how the PC evolves in real time. On the contrary, *ex situ* techniques measure the PC after isolation of the NPs–PC complex from the physiological environment.¹⁵ In this contest, different isolation methods could be applied, depending on the experimental requirements. The most common used methods are:

- **Centrifugation.** Based on the different densities of nanomaterials relative to free proteins, centrifugation is, to-date, the most widely used method for isolation of the PC around nanomedicines.^{22–25} Centrifugation is a simple and quick isolation method and an efficient way to retrieve enough proteins for their safe identification using mass spectrometry analysis, as the quality of identification is strictly dependent on the available amount of material.
- **Size exclusion chromatography (SEC).** In order to isolate the corona in a less perturbing manner, SEC was recently proposed as an alternative to centrifugation. This technique separates NP–PC complexes from unbound proteins through a column containing a porous stationary phase. Separation takes place since NP–PC

complexes are larger than the stationary phase pores, do not penetrate into the pores, and elute before the unbound proteins, which on the contrary can enter the pores and require a longer time to pass through the column.

This isolation method is less disruptive than centrifugation and weakly bound proteins may still be retrieved after the separation.^{17,20,26}

- **Magnetic separation/magnetic flow field fractionation (MgFFF).** This particular technique is based on the elution of magnetic NPs by means of a chromatography-like method in which the separation is carried out in a single liquid phase. MgFFF is characterized by the use of an external magnetic field applied perpendicularly to the direction of sample flow through an empty and thin ribbon like channel.²⁷ As demonstrated by Ashby et al., this method allows the screening of proteins with distinct exchange kinetics in the corona around NPs. In fact, MgFFF provides for a separation in non-equilibrium conditions able to cause continuous dissociation of the protein–NP complexes inside the column; that way, the dissociated proteins are constantly washed away from the complexes by the protein-free mobile phase.¹⁸

Analysis of the PC Structure

- **Dynamic light scattering (DLS).** DLS allows the determination of the hydrodynamic diameter of colloidal particles and conjugates. Therefore, DLS measurements are useful to determine changes in the diameter of NPs before and after incubation in a biological environment.²² Several studies employed the DLS technique aiming to evaluate the extent of PC formation, and to correlate an increase in NP diameter after exposure to serum or plasma to the formation of a

PC around the particle.^{24,25,28} The main advantage of DLS is the possibility to be used both *in situ* and after isolation of the NP-PC complex. However, in order to give reliable results, DLS measurements require a monodisperse population of NP-PC complexes with homogeneous shapes as it could strongly affect the hydrodynamic diameters. Recently, a very elegant approach on NP-PC complex size determination was given by Schmidt and co-workers²⁹; in this paper, the aggregation dynamics as well as the impact of different chemico-physical properties of NPs on the PC-NP complex size were analyzed.

- **Differential centrifugal sedimentation (DCS).** DCS is able to separate the components of a mixture on the basis of their density and size, as larger and denser objects require lower centrifugal forces to sediment. DCS allows the size distribution measurements of NP-PC complexes also *in situ*, but limits may also be present. In fact, this technique forces the samples to be repeatedly centrifuged followed by removal of the pellet and repeated with increased centrifugal force. Moreover, this technology may risk exposing the samples contaminations and poor recovery. This method was applied to determine differences in size between bare and corona-coated NP systems.^{24,25}
- **Transmission electron microscopy (TEM).** TEM is used to obtain images of the NPs before and after incubation in a biological fluid with the scope of determining the thickness of PC around the NPs. However, this technique requires a sample preparation, which may affect the morphology of NP-PC complexes.²⁵ In addition, counterstaining is required, since the small size of the NPs and the thin protein layer may provide insufficient contrast.³⁰

Protein Quantification

- **Bicinchoninic acid (BCA) assay.** This test combines the reduction of Cu^{2+} to Cu^{1+} by peptide bonds of the protein in alkaline solution with the selective colorimetric reaction of BCA-Cu^{1+} able to form a purple complex featured by absorption at λ 562 nm.³¹ In the case of PC-NP complexes, the BCA assay is performed to determine the total amount of proteins adsorbed onto NPs after incubation in plasma.³²⁻³⁴ Advantages of this technique are represented by its compatibility with several

reagents or buffers present in the samples and the limited amount of sample required for the analysis. However, the reaction is time and cost expensive as the unit cost is higher than for other colorimetric methods, such as the Bradford assay.³¹

- **Bradford assay.** This test detects proteins on the basis of their binding to Coomassie brilliant blue, forming a protein-dye complex with a change in the solution color from red to blue, due to a shift in the peak absorbance of the dye from λ 465 nm to λ 595 nm.³¹ As well as BCA assay, Bradford assay is employed in the determination of the amount of adsorbed proteins onto NPs.^{35,36} This colorimetric method is highly sensitive, quick and requires minimal amounts of sample for the analysis. In addition, it represents one of the less expensive colorimetric methods for protein quantification.
- **Thermogravimetric analysis (TGA).** This technique is commonly used to measure the amount of weight variation occurring after a thermodecomposition reaction in organic or semi-organic materials. Thus, the overall mass of the proteins adsorbed onto inorganic NP-surface can be determined by the loss of weight after the decomposition reaction.³⁷

Binding Affinity/Stoichiometry and Protein Interaction

- **Fluorescence correlation spectroscopy (FCS).** This technique provides information on both kinetic and thermodynamic properties of fluorescent molecules in solution, exploiting the temporal relaxation of the measured fluorescence fluctuations and the amplitudes of the fluctuations, respectively.³⁸ Thus, FCS experiments allow us to measure binding curves by exposing NPs in nanomolar dilutions to a wide range of protein concentrations and, thereby, yield information on the tendency of the protein to adsorb.³⁹
- **Size exclusion chromatography (SEC).** This technique allows determination of the affinity and lifetime of the NP-protein interaction. Ideally, the separation of proteins and other compounds by SEC is based on the size of the analytes in solution. Generally, the pore size and/or geometry restrict access of molecules based on their Stokes radius. The largest molecules/structures, which are excluded from the pores, elute first. Subsequent molecules elute in

1 order of decreasing size.⁴⁰ In the case of the PC,
2 if proteins exchange slowly from the particle,
3 they will elute rapidly with the particles, while
4 if the exchange is fast, the protein will elute at
5 the same time as without particles.²⁰

- 6 • *Isothermal titration calorimetry (ITC)*. This
7 method can be applied to measure the stoichi-
8 ometry, affinity and enthalpy of NP–protein
9 interaction. In this technique, protein is added
10 to a NP suspension in the sample cell, and the
11 difference in heat needed to keep both the sam-
12 ple and reference cells at the same temperature
13 is measured. If the concentrations of both NPs
14 and added protein are known, this technique
15 provides information on the number of bound
16 protein molecules per particle, the apparent
17 affinity and the enthalpy change.¹⁷
- 18 • *Surface plasmon resonance (SPR)*. SPR provides
19 information on the adsorption kinetics. In this
20 technique, NPs are anchored on the gold sur-
21 face of the sensor chip, and proteins are injected
22 to flow over the NP-modified surface. SPR mea-
23 sures the change of oscillation of surface plas-
24 mon waves that are caused by the adsorption of
25 molecules onto the metal surface.^{17,41,42}
- 26 • *Quartz crystal microbalance (QCM)*. This tech-
27 nology, based on the piezoelectric effect, mea-
28 sures the resonant frequency shift correlated to
29 mass changes at the oscillating quartz surface.
30 Either proteins or NPs are immobilized onto a
31 gold surface located on a quartz crystal; the
32 binding partner is injected into the flow-cham-
33 ber, passed over the quartz surface and the fre-
34 quency monitored in real-time. Real-time and
35 quantitative NP–protein binding profiles are
36 obtained, and the association and dissociation
37 constants can be determined by fitting to the
38 Langmuir adsorption isotherm.⁴³
- 39 • *Z-potential measurement*. Zeta potential is
40 another approach for the screening of NP–
41 protein interactions. Adsorbed proteins change
42 the zeta potentials and the isoelectric points
43 (IEP) of the particles, and the amount of the
44 adsorbed protein on particle surfaces could be
45 correlated with the zeta potential.²²
- 46 • *Computer simulation*. Beside the experimental
47 techniques, computer or *in silico* simulation of
48 NP–protein interactions is another possible
49 strategy to predict PC characterization and
50 composition. In fact, simulation provides infor-
51 mation on protein orientation and conforma-
52 tion with high spatial and temporal resolution
53 and it is applied to study protein adsorption to

54 NPs as function of surface ligand structure, sur-
55 face curvature and protein identity.¹⁵

Protein Conformation

- 62 • *Circular dichroism (CD) spectroscopy*. CD
63 measures the spectra of different protein sec-
64 ondary structures, as they possess their own CD
65 spectra in the UV region.^{44–46} This technique
66 can provide information on protein structural
67 changes resulting from the interaction with
68 NPs, but requires relatively high concentration
69 of the sample and cannot be applied to complex
70 protein mixtures.⁴¹
- 71 • *Fourier-transform infrared (FTIR) spectroscopy*.
72 Similar to CD spectroscopy, FTIR allows
73 the determination of conformational changes of
74 proteins. The protein secondary structures are
75 estimated on the basis of the absorption of
76 amide bonds. Among the amide I, II, and III
77 bands, the amide I vibrational band
78 (1700–1600 cm⁻¹) is the most sensitive and fre-
79 quently used to determine protein conforma-
80 tion.⁴¹ The FTIR method allows the detection
81 of NP–PC complexes already at a very early
82 stage as well as highlight conformational
83 changes during the ongoing aggregation
84 process.
- 85 • *Raman spectroscopy (RS)*. As with FTIR, RS
86 investigates the vibrational modes of molecules,
87 giving complementary information. Raman
88 spectra of proteins consist of bands associated
89 with the peptide main chain, aromatic side
90 chains, or sulfur containing side chains. Gener-
91 ally, RS is preferred to measure the protein–NP
92 complexes in aqueous solution; moreover,
93 Raman spectra are more simple than IR spectra
94 since the localized vibrations of double or triple
95 bonds or electron-rich groups produce more
96 intense bands than the vibrations of a single
97 bond or electron-poor groups.⁴¹
- 98 • *Nuclear magnetic resonance (NMR) spectroscopy*.
99 As is well known, the phenomenon of
100 nuclear magnetic resonance can provide
101 detailed information about the structure,
102 dynamics, reaction state, and chemical environ-
103 ment of molecules. The application of NMR to
104 PC characterization allowed residue-specific
105 structural information regarding the adsorbed
106 protein to be obtained. In particular, localized
107 conformational information was obtained
108 regarding some adsorbed peptides, especially by
109 means of solid-state NMR.⁴⁴

- *Differential scanning calorimetry (DSC) spectroscopy*. DSC measures the heat change associated with the thermal denaturation of a molecule when heated at a constant rate. In this way, DSC measures the enthalpy change (ΔH) of unfolding that results from heat-induced denaturation. Thus, information on protein stability after the NP-adsorption process can be highlighted.⁴⁷
- *Fluorescence correlation spectrometry (FCS)*. This technology can be used to get information about the protein conformation since the maximum level in fluorescence emission spectrum intensity changes correspondingly to the protein conformation.⁴⁸

Composition

The identities of the proteins composing the corona around NPs can be investigated using techniques such as gel electrophoresis [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2-DE)] and mass spectrometry (MS).

It must be underlined that these techniques can be performed only *ex situ*, after isolation of the NP-PC complexes from excess plasma or serum.

- *One-dimensional gel electrophoresis (1-DE or SDS-PAGE)*. In SDS-PAGE, the protein mixture is separated depending on molecular weights after exposure to an electric field. The proteins migrate through a polyacrylamide gel and are separated according to their size due to their different electrophoretic mobilities. Proteins must be previously denatured and negatively charged by an anionic detergent (SDS). After the migration, the proteins can be stained using different methods, such as Coomassie brilliant blue or silver nitrate staining. Densitometry analysis can be performed in order to quantify protein abundance. Molecular weights of separated proteins can be extrapolated by comparing the position of the protein bands with SDS-PAGE profile of a protein molecular weight marker. This technique is often followed by mass spectrometry analysis to determine the identities of the separated proteins.⁴¹
- *Two-dimensional gel electrophoresis (2-DE)*. This technique separates protein samples in two steps or dimensions. In the first dimension of 2-DE, named isoelectric focusing (IEF), proteins are separated accordingly only to their IEP. In

the second dimension, SDS-PAGE, proteins are fractionated on the basis of their molecular weights. The bands are then visualized through a staining method and analyzed for protein quantification.⁴⁹ This technique also allows protein identification, since a 2-DE gel can be compared to the 2-DE map of proteins.^{32,33,49,50}

- *Mass spectrometry (MS)*. MS has been widely applied to identify the proteins of the corona.

In protocols present in the literature,^{51–53} proteins need to be first digested into smaller peptides with a proteolytic enzyme such as trypsin, in order to reduce the size of the analytes and to produce more suitable data in agreement to the mass range of the instrument. These peptides are ionized in the ion source and then introduced into a region of high vacuum. Ions are separated in function of their mass to charge ratio (m/z) under either a strong electromagnetic field or in a long drift tube. The resulting mass spectra allow the primary sequence of each given peptide in the mixture to be determined. These data are then compared against the database of the species used in the experiment to recover the protein identities.⁴¹ With this procedure, MS was applied to identify NP PCs using gel- and non-gel-based methodologies.

Gel-based techniques require, as first step, a protein separation on SDS-PAGE: in more details, the bands of interest are cut from the gel and digested by trypsin, and then the peptides are analyzed by mass spectrometry. This technique was widely employed in order to determine the protein pattern of the whole PC around NPs.^{23,24,54}

On the other hand, the *non-gel-based method* can be applied either on proteins still adsorbed onto the NPs or after protein desorption. The proteins are digested by trypsin and the resulting peptides are directly analyzed by means of MS. Before trypsin digestion, protein denaturation is always performed in order to make the domain for trypsin more accessible.

Overall, both non-gel and gel-based methods require separation of the peptides before the MS injection, exploiting, for example, liquid chromatography.⁴¹ Several approaches were therefore proposed to this aim: *nanoscale liquid chromatography-quadrupole time-of-flight MS/MS* (nLC Q-TOF MS/MS), *nanoelectrospray liquid chromatography-tandem mass spectrometry* (nLC-MS/MS), *nano-liquid chromatography MALDI-TOF/TOF*, *ion trap-mass spectroscopy* (IT-MS) and *matrix-assisted laser desorption/ionization time-of-flight secondary ion mass spectrometry* (MALDI-TOF-SIMS).^{23,24,26,36,55–57}

TABLE 2 | Schematic Illustration of the Main Techniques Used for the Characterization of Different PC-Related Parameters

Parameter	Technique(s)
Structure/thickness	Dynamic light scattering (DLS)
	Differential centrifugal sedimentation (DCS)
Protein quantification	Transmission electron microscopy (TEM)
	Bicinchoninic acid (BCA) assay
	Bradford assay
Binding affinities/stoichiometry and NP–protein interaction	Thermogravimetric analysis (TGA)
	Fluorescence quenching titration
	Fluorescence correlation spectroscopy (FCS)
	Size exclusion chromatography (SEC)
	Isothermal titration calorimetry (ITC)
	Surface plasmon resonance (SPR)
	Quartz crystal microbalance (QCM)
Protein conformation	Zeta potential (Z-pot)
	Computer simulation
	Circular dichroism (CD)
	Fourier-transform infrared (FTIR) spectroscopy
	Raman spectroscopy (RS)
	Nuclear magnetic resonance (NMR)
PC composition	Differential scanning calorimetry (DSC)
	Fluorescence correlation spectrometry (FCS)
	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)
	2D-PAGE
	Mass spectrometry (in-gel method)
	Mass spectrometry (non-gel method)

NP, nanoparticle; PC, protein corona.

In summary, the discussed techniques for the investigation of the PC are listed in Table 2.

TECHNICAL APPLICATIONS IN HARD CORONA STUDY

Table 3 summarizes the identification of the HC features for different NPs using the methods described in the previous section. From a technological point of view, remarkably, it must be highlighted that HC analysis necessary requires an *ex situ* approach. As a first step, the most used method for HC isolation is the centrifugation, as illustrate in Figure 1.² Generally, it allows the isolation of NP–HC complex, while weakly bound proteins are lost. However, it is important to note that the duration of washing as well as the solution volumes used during the washing steps could impact the final results, and that the most abundant proteins, or protein aggregates, may be recovered after sedimentation at the bottom of the centrifugation tube due to in-correct washing.^{2,15} Only a limited number of papers described the application of different isolation methods such as gel filtration,⁷⁰ size exclusion chromatography,¹⁷ and magnetic separation^{14,18,70} in order to characterize the HC.

HC Structure and Quantification

As reported in the literature, after the removal of the SC, it is possible to analyze the HC in terms of thickness or increase in mass percentage.^{23,58,71} The evaluation methods are almost the same independently of the kind of NPs (i.e., inorganic or organic polymeric NPs). For example, Casals et al. noted that the diameter of gold NPs (initially 10 nm) changed when incubated in fetal bovine serum. In particular they highlighted that a long incubation time (48 h) lead to a stable protein coating on the NP surface, which produced an increase in diameter of more than 50% in respect to the initial diameter.²² On the other hand, Monopoli et al. demonstrated that the thickness of the HC in polystyrene NPs could change on the basis of plasma concentrations used in the experiment: a higher plasma concentration leads to a thicker HC (38% hydrodynamic diameter increase).²⁴ This observation was confirmed by Caracciolo et al. in the case of 1,2-dioleoyl-3-trimethylammonium propane/DNA NPs incubated in a concentrated solution of plasma, which led to the detection of a thicker total PC (31% of hydrodynamic diameter increase).⁷² All of these measurements were obtained by DLS analysis, the most applied technique in order to identify HC thickness.

TABLE 3 | Overview on the Technical Applications Employed for HC Characterization

Type/Material	Size (nm) Z-Pot (mV)	Incubation Medium	Isolation Method	(i) Thickness	Characterization Method of HC Applied to:				References
					(ii) Protein Quantification	(iii) Binding Affinity/ Protein Stoichiometry/ Protein Interaction	(iv) Protein Conformation	(v) Composition	
Gold	10 –45	Cellular medium with 10% FBS	centrifuge	DLS	/	/	/	LC-MS	Casals et al. ²²
Polystyrene (P), silica (S)	200 –25	HP (different conc)	centrifuge	DLS/DCS	/	/	/	SDS-PGS and LC-MS	Monopoli et al. ²⁴
Gold	22–26 –30	Cellular medium with 10% FBS	centrifuge	DLS	/	/	/	/	Wang et al. ⁵⁸
Gold	30–50 –33/–38	HP	Centrifuge	DLS/ TEM/AFM	/	/	/	2D-PAGE, IT-MS	Dobrovolkaia et al. ⁵⁵
Silica	70–80–250–500–900 –12/–37	Cellular medium with 10% FBS	centrifuge	TGA	TGA	/	/	SDS-PAGE, LC- MS	Clemments et al. ³⁷
Polystyrene	50–100 +23/–32	HP	Centrifuge	/	NP-HC complex weight	/	/	SDS-PAGE, LC- MS	Lundqvist et al. ²³
Polystyrene/ silica	50, 100, 200 /	HP	Centrifuge	DLS, DCS, TEM	/	/	/	/	Walczyk et al. ²⁵
PLGA	227 –20	HP	Centrifuge	/	BCA	/	/	LC-MS	Sempf et al. ⁵⁷
SPION	600/900 –10/–40	Culture medium	Centrifuge	TEM	Bradford (surfactants)	FTIR	/	MALDI-TOF- SIMS	Mbeh et al. ³⁶
Gold	10 +25	Cell lysates	Centrifuge	DLS	Bradford	/	/	Western blot, LC-MS	Arvizo et al. ³⁵
Gold	30, 90 /	α-Synuclein HEPES buffer	Centrifuge	DLS	UV-vis measurement	FCS	/	LC-MS	Yang et al. ⁵⁹
Polystyrene	50, 100 /	HP	/	/	/	FCS	/	/	Milani et al. ⁶⁰
Hydroxyethyl starch	200/270 –30	HP, HSA and Apo A-I protein solution	centrifuge	DLS	Quantification kit	ITC	/	SDS-PAGE	Winzen et al. ⁶¹
Silica	100, 200, 270 +2, –25, –35	BSA solution	Centrifuge	TEM	/	Z potential measurement (time evolution measurements)	/	/	Natte et al. ⁶²
Polystyrene	60 +20/–30	BSA solution	/	/	ITC	ITC	CD	/	Fleischer and Payne ¹¹
Gold	40 /	BSA solution	/	/	/	FCS	CD	/	Wangoo et al. ⁶³

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TABLE 3 | Continued

Characterization Method of HC Applied to:									
Type/Material	Size (nm) Z-Pot (mV)	Incubation Medium	Isolation Method	(i) Thickness	(ii) Protein Quantification	(iii) Binding Affinity/ Protein Stoichiometry/ Protein Interaction	(iv) Protein Conformation	(v) Composition	References
SPION	5, 8 /	Human transferrin solution	Magnetic separation	TEM	/	/	CD	/	Mahmoudi et al. ⁶⁴
Silica	6, 9, 15 /	HCAI protein solution	gel permeation chromatography	/	UV adsorption	/	NMR, near UV-CD	/	Lundqvist et al. ⁴⁴
Silica	25,225	HSA, HSF, HGG protein solutions	Centrifuge	/	BCA (surfactants)	/	FTIR	/	Ma et al. ⁶⁵
Alumina	100/300 /	BSA, LZM, FBG protein solutions	Centrifuge	/	UV adsorption (surfactant)	/	DSC, FTIR	/	Brandes et al. ⁴⁷
Silica	285 /	BSA, HEL, RNase, LPO	Centrifuge	/	/	FTIR, Z potential measurement (time evolution measurements)	RS	/	Turci et al. ⁶⁶
Titanium oxide	20 /	Tubulin protein solution	/	/	/	/	FCS	/	Gheshlaghi et al. ⁶⁷
NIPAM:BAM copolymer	70/700 /	HP	Centrifuge	/	/	ITC	/	SDS-PAGE, LC- MS	Cedervall et al. ¹⁷
Cyanoacrylate	140 -20	Rat serum	Centrifuge	/	/	/	/	2D-PAGE, Western Bolt	Kim et al. ⁶⁸
Silica, polystyrene	30/140 -30/+50	HP	Centrifuge (through a sucrose cushion)	DLS	/	/	/	SDS-PAGE + immunoblot, DIA-MS	Tenzen et al. ⁶⁹ , Docter et al. ⁵¹

/, range of values; AFM, atomic force microscopy; Apo A-I, apolipoprotein A-I; BCA, bicinchoninic acid assay; BSA, bovine serum albumin; CD, circular dichroism; DCS, differential centrifugal sedimentation; DIA-MS, data-independent acquisition mass spectrometry; DLS, dynamic light scattering; DSC, differential scanning calorimetry; FBG, bovine serum fibrinogen; FCS, fetal bovine serum; FCS, fluorescence correlation spectroscopy; FTIR, Fourier-transform infrared spectroscopy; HCAI, human carbonic anhydrase-I; HEL, hen egg lysozyme; HGG, human gamma globulin; HSA, human serum albumin; HSF, human serum fibrinogen; ITC, isothermal titration calorimetry; IT-MS, ion trap-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; LPO, lactoperoxidase; LZM, hen's egg lysozyme; MALDI-TOF-SIMS, matrix-assisted laser desorption/ionization time-of-flight secondary ion mass spectrometry; NIPAM:BAM, N-isopropylacrylamide/N-tert-butylacrylamide; NMR, nuclear magnetic resonance; PLGA, poly-lactic-co-glycolic acid; RNase, bovine pancreatic ribonuclease A (RNase); RS, Raman spectroscopy; SPION, super paramagnetic iron oxide nanoparticles; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TGA, thermogravimetric analysis; Z-pot, zeta potential; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

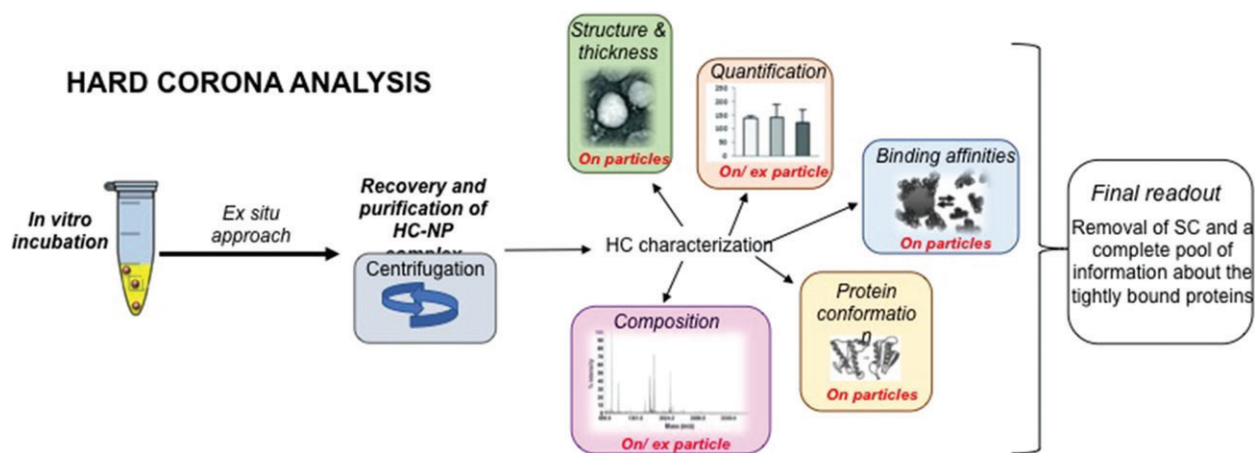


FIGURE 1 | Schematic illustration of hard corona (HC) studies. This kind of analysis requires an ex situ approach (generally by centrifugation). After this first step, the HC characterization is continued with different analytical pathways. Data collected from all these investigations could give a complete pool of information about HC features.

However, it is worth mentioning that centrifugation cycles, performed in order to isolate the HC–NP complex, could lead to aggregation phenomena. Inevitably, these aggregates could dramatically affect the results obtained by DLS and the final thickness should not be considered as comprehensive of the whole sample. As an example, Wang and colleagues observed a change in both size dimension and distribution of gold-NPs after serum incubation; particularly, the average diameter of NPs dramatically increased from 25 to 83 nm and distribution become more heterogeneous. They hypothesized that the increase in NP diameter was not only due to the formation of HC, but also to the presence of NP–protein agglomerates, caused to the presence of Ca^{++} and Mg^{++} ions present in the medium.⁵⁸

To complement the information given by DLS, TEM analysis can be applied to furnish structural data, as reported by Walczyk et al.²⁵ The authors compared polystyrene NPs before and after plasma incubation, evaluating the dimensions of about 500 NPs for each kind of sample (bare and protein-coated NPs) to reach statistically consistent results. The DLS results indicated that that after plasma incubation, the shell thickness values increased roughly 5–10 nm, but TEM images did not give the same output, as also reported by Dobrovolskaia et al. using gold NPs.⁵⁵ This difference was probably due to the different technology applied; TEM measures NP size on a grid support while DLS evaluate the hydrodynamic diameter of NPs in suspension. However, in this case, TEM analysis was considered useful to prove that plasma incubation did not change the agglomeration state of the NPs and, as a consequence, that DLS analysis was not affected by particle agglomeration phenomena.⁵⁵ This shows

the importance that attention must be given to the experimental conditions and especially to the analytical times.⁷³

Moreover, the extent of protein coating forming the HC can be expressed not only as ‘thickness values’, but also quantitatively. Generally, colorimetric assays are employed (i.e., BCA and Bradford) to measure the HC protein amount on NPs^{36,57} or, inversely, the non-adsorbed proteins left in the medium.³⁵

Alternatively, Clemments et al. used TGA analysis to characterize the mass percentage of the HC on their spherical dense/mesoporous silica NPs. In this case, the total amount of adsorbed protein was calculated as a function of weight loss. The data results and reliability of these results are still debatable. In fact, as expected, the smallest particles were found to adsorb the greatest amount of protein, due to the greater surface area (when equal weights of NPs were used). However, by normalizing the total amount of adsorbed protein to the total surface area of each sample, the results clearly stated that an increase in particle diameter greatly increased the amount of adsorbed protein. Thus, the authors hypothesized that a decreased surface curvature of larger particles could favor protein binding, as proteins are able to pack together more closely.³⁷

Binding Affinities/Stoichiometry and NP–Protein Interaction

Data related to the layer thickness of the HC is frequently reported in scientific researches.^{22,24,25,55,58,72,74,75} On the contrary, the absolute number of bound

1 proteins and their exchange dynamics in body fluids
2 are difficult to be assessed with standardized
3 protocols.

4 In particular, regarding the HC, we are limited
5 by a lack of information on the dynamics of protein
6 exchange, mainly due to a shortage of techniques
7 that allow the assessment of the binding and unbind-
8 ing of specific proteins to NPs.⁶⁰ To overcome this
9 drawback, Yang et al. elaborated on a two-step fluo-
10 rescence quenching experiment aiming to quantify
11 the binding affinity of the HC for gold-NPs incu-
12 bated with a single protein solution [α -synuclein
13 (α -syn)].⁵⁹ Briefly, in the first step, different concen-
14 trations of gold-NPs were titrated against a known
15 concentration of α -syn obtaining the first fluores-
16 cence quenching plot. Coated gold-NPs used in this
17 first titration were then collected, purified
18 (by centrifugation) and used in the second titration
19 set against the same amount of α -syn (second fluores-
20 cence quenching plot). Authors assumed the fluores-
21 cence quenching obtained in this second step was the
22 combination of the gold-NPs and SC light absorp-
23 tion. The difference between the first and second flo-
24 rescence plots was then due solely to the HC of α -syn
25 on gold-NPs and it was used to calculate HC binding
26 constant.

27 Alternatively, Milani et al. used FCS to measure
28 HC binding rate overtime in terms of the number of
29 transferrin molecules bound per particle to sulfonate
30 (PSOSO₃H) and carboxyl- (PSCOOH) polystyrene
31 NPs. The authors found that the fraction of mole-
32 cules (proteins) bound to the NPs could be described
33 with a universal adsorption curve if plotted as a func-
34 tion of molar protein-to-NP ratio. In particular, this
35 adsorption curve was characterized by a two time-
36 scale dynamic due to a first strongly bound mono-
37 layer (namely HC) and to a second weakly bound
38 layer (namely SC). Thus, they demonstrated that the
39 HC was characterized by an off rate longer than the
40 experimental time scale of a few hours, while the SC
41 appears to exchange proteins within minutes under
42 buffered conditions.⁶⁰

43 The binding affinity or the exchange rate of the
44 proteins belonging to PC are generally investigated in
45 a comparative manner; this method does not imply a
46 clear distinction between HC and SC, but it allows
47 bound proteins to be ordered on the basis of their
48 affinity to the NPs. As an example, a recent study by
49 Winzen and co-workers applied ITC to characterize
50 PC binding affinity around hydroxyethyl starch
51 nanocapsules. Results revealed large amounts of
52 human serum albumin (HSA) amount present with
53 low binding affinity, probably ascribable to the SC;
54 on the contrary, apolipoprotein A-I was present in

small amounts but with high binding affinity, typi- 57
cally considered as a HC component.⁶¹ 58

59 In addition, there have also been a huge num-
60 ber of studies evaluating NP-protein dynamics, which
61 provided information not necessarily related to the
62 binding affinity constants. One example assessed the
63 dynamics and evolution of the PC-NPs at different
64 incubation times by evaluating zeta potential
65 values,⁶² zeta potential and QCM,⁴³ or by zeta
66 potential and SPR analysis.²² In the latter case,
67 depending on time of incubation, SPR measurements
68 shifted over time thus, revealing the formation of a
69 dense dielectric layer around gold NPs due to the
70 adsorption of proteins onto the NPs surface.²² 70
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74 Protein Conformation in the HC

75 Curved NP surfaces in comparison with planar sur-
76 faces are known to be able to provide extra flexibility
77 and enhanced surface area to the adsorbed protein
78 molecules.⁷⁶ However, only in the recent years has
79 the attention focused on the impact of different NPs
80 surfaces ‘architectures’ on protein conformation. In
81 particular, curved NP surfaces were demonstrated to
82 affect the secondary structures of proteins, and, in
83 some cases, causing irreversible changes.⁷⁷ This phe-
84 nomenon is particularly relevant when considering
85 the biological fate of NPs, due to obvious implica-
86 tions for clearance and immunological responses.
87 Thus, a number of studies have attempted to investi-
88 gate on the conformational changes of the proteins
89 adsorbed onto NPs. All the studies referred to the
90 proteins composing the HC layer, the structure clo-
91 sest to nanomaterial, which are affected by modifica-
92 tions of secondary structures in function of surface
93 changes. Aiming to use CD spectroscopy to investi-
94 gate the interaction of polystyrene NPs with cellular
95 receptors after adsorption of BSA, Fleischer and
96 Payne demonstrated that the secondary structure of
97 adsorbed BSA is strongly responsible for the interac-
98 tion of the complexes with the receptors.¹¹ Also,
99 Wangoo et al. performed CD experiments and found
100 that BSA undergoes to conformational changes in a
101 dose dependent manner when incubated with gold
102 NPs.⁶³ Mahmoudi et al. used the same technology to
103 study the interactions of iron saturated human trans-
104 ferrin protein with both bare and polyvinyl alcohol-
105 coated superparamagnetic iron oxide nanoparticles
106 (SPIONs).⁴¹ In this case, the exposure of human
107 transferrin to SPIONs led to a protein conforma-
108 tional change, from a closed to open conformation,
109 causing the release of iron by the protein. This new
110 conformational state was also maintained after the

removal of the magnetic nanoparticles indicating the changes in transferrin structure were irreversible.⁴¹

As a general consideration, CD technology can be considered a powerful analytical tools in determining the protein conformation in solution or when adsorbed onto other structures. This is confirmed by the large number of studies reporting CD spectroscopy to study protein conformational changes. However, data coming from CD analysis can be supported by other analytic methods such as NMR.⁴⁴ For example, Lundqvist et al. studied the conformational change of the protein HCAI adsorbed onto the surface of silica NPs. Through NMR and near-UV CD, the authors were able to demonstrate that longer incubation times correlated with a gradual shift of the native HCAI to a more disturbed conformational form.⁴⁴

Another technique often employed in the determination of protein conformation is represented by FTIR. In a recent work, Ma et al.⁷² used FTIR to investigate the adsorption of human albumin (HSA), globulin (HGG), and fibrinogen (HSF) onto different kinds of mesoporous silica nanoparticles (MSNs). The authors found that the conformation of adsorbed HSA and HSF is affected by the pore size and morphology of their MSNs; on the contrary, HGG conformation was not affected by adsorption. Moreover, these conformational changes of the adsorbed proteins were able to affect the saturated adsorption capacity of the NPs.⁶⁵ In another research, the FTIR method was employed in association with highly sensitive DSC to determine adsorption-induced structural changes of the same model proteins [BSA, lysozyme (LZM) and fibrinogen (FBG)] on different ceramic nanoparticles. In almost all cases, protein adsorption resulted in destabilization and structural loss of the bound proteins. In particular, a loss in α -helical structure seemed to be the most sensitive structure on adsorption-induced rearrangements. Moreover, the authors conclude that the two techniques applied in the study (DSC and FTIR spectroscopy) were able to provide complementary information on adsorption-induced structural changes. Specifically, DSC was identified as the most suitable technique in order to provide information about the molecular level (thermal stability, overall structure) while FTIR gave relevant information on the sub-molecular level (secondary structure).⁴⁷

Alternatively, Raman spectroscopy (RS) can be used to evaluate the occurrence of conformational changes. Recent experiments revealed that a significant shift of the amide-I band could be observed after incubating silica NPs with a BSA protein solution, whereas, other model proteins maintained their

native conformations, after adsorption onto the surface of the NPs (RNase and HEL), under the experimental conditions employed.⁶⁶

Apart from the structural information obtained by CD, FTIR, NMR and RS analysis, some indications about protein conformation changes can be achieved by using FCS. Some authors exploited FCS to investigate the effect of titanium dioxide (TiO₂) NPs on microtubules polymerization since the tubulin is able to produce a fluorescence quenching and a blue shift of the maximum emission wavelength after the incubation with TiO₂ NPs. As evidence, the authors concluded that TiO₂ NPs were able to inhibit tubulin polymerization, thus confirming that NPs lead to protein function alteration by inducing changes in protein folding.⁶⁷

HC Composition

In order to detect the composition of the HC, after isolating the NP-HC complex from the excess of protein in the media, a preliminary desorption process of the proteins from the nanomaterial surface, generally named 'ex-particle' protocol, can be required. Protein desorption from the nanomaterial can be performed by treating the HC-NPs complex with high temperatures, high salt concentrations or detergents to detach them from the complex and make them suitable for the analysis (protein electrophoresis or enzymatic digestion followed by MS). Alternatively, the 'on-particle' protocol can be adopted to by-pass the desorption procedure, but it requires an enzymatic digestion performed on NP surface. This method is particularly useful when the strength of the interaction between the protein and the nanomaterial could cause a partial detachment during desorption leading to unsatisfactory results.

Generally, the protein desorption method of choice must take great account into the final aim, the technical procedures which are compatible with the samples, the raw materials and the experimental features.

Sempf et al.⁵⁷ chose to apply an 'on particle' approach to the analysis of the HC formed on polylactic-co-glycolic acid (PLGA)-NPs after incubation in human plasma. The proteins were directly digested on the NP surface using trypsin and then analyzed by nLC MALDI-TOF/TOF (without gel analysis).⁵⁷ The authors identified 15 proteins in the HC, 7 of which were not typically abundant in plasma. Moreover the authors compared their results with those obtained by other authors⁴⁹ using other methodologies to investigate the HC of PLGA-NPs. The results were strikingly different within the two experimental sets,

1 particularly regarding the presence of proteins such
2 as albumin, Apo A-1, Apo A-4, Apo C-3, and trans-
3 ferrin. Maybe, as explained above, these differences
4 could be ascribable not only to the features of the
5 NPs (size, surface curvature, etc.) but also to the
6 applied analytical method (on-particle vs ex-particle
7 digestion/in-gel vs non-gel approach).

8 Other papers described the combined method
9 of SDS-PAGE followed by MS^{23,24,37}; one of the
10 most complete work dealt with the detection and
11 evaluation of the HC of NIPAM-BAM NPs with
12 varying sizes (70–700 nm) and polymers ratios,
13 finally identifying HSA, apolipoprotein A-IV, apoli-
14 poprotein A-I and apolipoprotein A-II as the most
15 consistently present proteins composing the HC
16 around these NPs.²⁶ Another research group
17 employed 2D-PAGE and Western blotting analysis to
18 compare the HC profile of pegylated- polyhexadecyl-
19 cyanoacrylate (PHDCA) NPs to non-pegylated
20 PHDCA-NPs. The results revealed that, after incuba-
21 tion with rat serum, apolipoprotein E (ApoE)
22 adsorbed more onto PEG-PHDCA than on PHDCA
23 nanoparticles.⁶⁸

24 As also remarked by Walkey and Chan, some
25 proteins adsorb abundantly to every nanomaterial,
26 while other proteins do not. The abundant proteins
27 in the HC are not always the same and it is strictly
28 dependent on the NP feature and experimental condi-
29 tion adopted. It is also important to note that the
30 total number of unique proteins within the PC of any
31 nanomaterial is unknown. While LC-MS/MS is more
32 sensitive, and tends to detect more low abundance
33 proteins, neither PAGE nor LC-MS/MS is sensitive
34 to the single molecule level.¹⁵

35 Interestingly, a recent work proposed an up-
36 graded method by combining SDS-PAGE/MS to
37 obtain time-resolved HC profiles formed on various
38 NPs.⁵¹ Briefly, after NP incubation in protein contain-
39 ing medium, NP-protein complexes were rapidly sepa-
40 rated from unbound proteins by sedimentation
41 through a sucrose cushion, and washed to obtain the
42 HC-NP complex. Subsequently, the protein desorption
43 and separation could be obtained via 1D SDS-PAGE
44 in association with an immunoblot analysis to identify
45 and (semi)-quantify the presence of specific corona
46 proteins. Alternatively, the authors proposed a proto-
47 col based on protein desorption and digestion with
48 trypsin followed by resolution of the obtained peptides
49 by high-resolution nanoscale ultra-performance liquid
50 chromatography on reversed-phase (C18) columns,
51 analyzed by ion mobility-enhanced data-independent
52 acquisition (DIA) MS. This complex protocol could
53 give interesting improvements since the sucrose cush-
54 ion centrifugation method efficiently limits the contact

time of NPs with the biological fluid of interest, render-
ing analyses of short time periods feasible. Moreover,
the adapted label-free quantification by LC-MS (taken
by the recently described ion mobility-enhanced, DIA-
based label-free quantitative proteomics workflow of
Distler et al.)⁷⁸ allows reliable and highly reproducible
quantification of corona components. Moreover, the
authors specified that the protocol could be readily
extended to the investigation of PCs from various
nanomaterials, as confirmed by the application of this
protocol to different silica nanoparticles and polysty-
rene nanoparticles.^{51,69}

TECHNICAL APPLICATIONS IN SOFT CORONA STUDY

An overview of studies referring to SC characteriza-
tion is reported in Table 4. In comparison with the
HC, a limited number of methods for SC detection
are available. As a consequence, poor knowledge
concerning the SC is present.

The major drawback is the SC isolation. In fact,
the common isolation methods, inevitably, stress the
NP-PC complex resulting in a partial, or sometimes
total, detachment of the SC. As a matter of fact,
almost a totality of studies on the SC relied upon *in*
situ techniques (previously described) and is mostly
focused on the identification of the SC structure, with
the exclusion of a few exceptionally complex experi-
mental procedures (Figure 2). To-today SC character-
ization still represents an intriguing challenge.

SC Structure and Quantification

The major part of studies concerning the SC structure
provide the measurement of the total PC thickness
depleted of HC contribution. However, this indirect
measurement needs to be carefully evaluated in order
to avoid unreliable results and therefore, presents
several criticisms such as the congruity of time, con-
dition and methods of analysis for total PC and HC
thickness. In fact, it is obvious that the comparison
between *in situ* and *ex situ* measurements could pro-
vide only an approximation regarding the SC struc-
ture since it is not possible to compare measurements
performed in a suspension medium with a different
diffraction index, a concept often neglected in some
PC studies.²² Considering this gap, a clear study of
the SC structure and thickness only by DLS analysis
is not an easy thing to manage. Schaffler et al. tried
to by-pass this gap by incubating gold NPs in a
diluted serum solution (1:100 in PBS buffer).⁷⁹ With
this protocol, the measurements performed *in situ* are
more comparable with the measurements performed

TABLE 4 | Overview on the Technical Applications Employed for SC Characterization

Type/Material	Size (nm) Z-Pot (mV)	Incubation Medium	Isolation Method	(i) Thickness	Characterization Method of SC Applied to:				References
					(ii) Protein Quantification	(iii) Binding Affinity/Protein Stoichiometry/Protein Interaction	(iv) Protein Conformation	(v) Composition	
Gold	10 -45	Cellular medium with 10% FBS	/	DLS <i>in situ</i> (HC subtraction)	/	/	/	/	Casals et al. ²²
Gold	5, 15, 80 -30/-40	Diluted mouse serum	/	DLS <i>in situ</i> TEM	/	/	/	/	Schaffler et al. ⁷⁹
Polystyrene (P), silica (S)	100, 200 (P), 50 (S) -25/-50	HP	/	DCS	/	/	/	/	Walzyk et al. ²⁵
Polystyrene (P), silica (S)	200 -25	HP (different conc)	/	DCS	/	/	/	/	Monopoli et al. ²⁴
NIPAM-BAM copolymer	70 /	HDL suspension	/	/	Theoretic binding model confirmed by SPR	/	/	/	Dell'Orco et al. ⁸⁰
Hydroxyethyl starch	200/270 -30	HP, HSA and Apo A-I protein solution	/	DLS	/	ITC	/	/	Winzen et al. ⁶¹
NIPAM-BAM copolymer	7/700 /	HP, HSA protein solution	SEC	/	/	ITC	/	SDS-PAGE, LC-MS	Cedervall et al. ¹⁷
SPION	15/30 -30/-45	HSA and IgG depleted	F4	/	/	F4	/	LC-MS	Ashby et al. ¹⁸
SPION	18/38 -26/+36	FBS	Magnetic separation	/	/	/	/	SDS-PAGE, LC-MS	Sakulku et al. ¹⁴

/, range of values; Apo A-I, apolipoprotein A-I; DCS, differential centrifugal sedimentation; DLS, dynamic light scattering; F4, flow field-flow fractionation; FBS, fetal bovine serum; HDL, high-density lipoprotein; HP, human plasma; HSA, human serum albumin; IgG, immunoglobulin G; ITC, isothermal titration calorimetry; LC-MS, liquid chromatography-mass spectroscopy; NIPAM-BAM, N-isopropylacrylamide/N-tert-butylacrylamide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SPK, surface plasmon resonance; SPION, super paramagnetic iron oxide nanoparticles; TEM, transmission electron microscopy; Z-pot, zeta potential.

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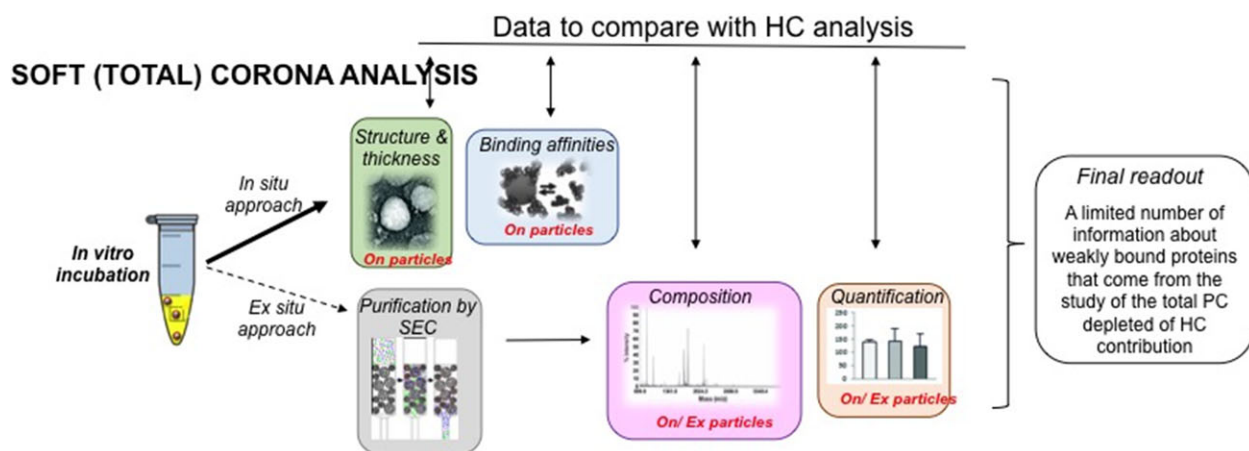


FIGURE 2 | Schematic illustration of soft corona (SC) studies. The major part of the studies are based on in situ approach (solid black arrow), with the exception of a few cases based on ex situ approach (dashed arrow). The information given by the SC analysis are more limited or completely missing in number if compared with HC analysis (if considering protein conformation analysis). Moreover, SC data are generally obtained by the measurement of the total PC subtracting the HC (as indicated by the double arrows in the upper part of the scheme).

after the centrifugation and re-suspension procedures. The data obtained from the incubation *in situ* experiment showed an increased hydrodynamic diameter of the gold NPs; this increase remained unchanged even after centrifugation and re-suspension. The lack of size reduction after centrifugation is interpreted as a technical limit of DLS in measuring the labile part of the PC (*alias* SC), as only the HC was detected before and after the purification processes.

Thus, in this case, DLS analysis is not strongly affected by different incubation media diffusion indices of, but the incubation in diluted plasma could lead to an incomplete coverage of proteins around the NPs. In fact, total plasma incubation better simulates the *in vivo* conditions that give a large excess of proteins occurring to saturate the surface of NPs, while a deficit of protein concentration in the incubation medium can explain the behaviour of gold NPs.

DCS represents the other mainly elective method to study the SC; with respect DLS, the DCS technique is limited by the requirement of applying a mathematic model. For example, aiming to identify a reliable size by DCS, one must know the shape and internal density distribution of each aggregate. To overcome this problem, researchers present their data by correlating the equivalent diameters for spheres of homogeneous density and relative 'apparent' molecular weight.²⁵ Thus, the 'true' size of the NP–protein complex and the corona size were computed on monomeric NPs–protein complexes using a simple core–shell model of two densities (bare particle material density and adsorbed protein–biomolecule density). This core–shell model is generally the most used for these experiments.^{81,82} It is a simple model to analyze

data for shell-coated particles and to get an estimation of the shell thickness. In this case, the shell is represented by the PC. In this study, the PC of polystyrene NPs was measured under several different conditions including full plasma (diluted in PBS), after washing, centrifugation, and re-suspension in PBS buffer, thus enabling washing-off of the excess (unbound or loosely bound) proteins. In the presence of excess plasma, these different experimental conditions allowed the authors to draw connections between the NP–corona complexes *in isolation* and *in situ*, to finally refer to the presence of the SC. The DCS method was applied in the experiment on both silica and polystyrene NPs after plasma incubation.²⁴ Interestingly, after centrifugation no difference in NP size was reported, but only a reduction of the PC thickness, inversely proportional to the plasma concentration, was observed.

Overall, the experiments described above clearly showed that, independent of the NP characteristics (material composition, size) and incubation conditions (time and temperature), both techniques (DLS and DCS) are able to describe the increase in NP diameter after plasma incubation. However, discriminating between HC and SC is not always so clear. In opposition with Casal's work, Schaffler's group declared the impossibility to measure the SC by DLS.⁷⁹ Other researchers²⁵ tried to calculate the SC contribution in PC thickness by an indirect method in which the diameter of the HC, calculated by core–shell model, was subtracted from the total diameter is measured by DCS; in this experiment, data seemed to support the efficacy of DCS method for SC structure determination.

1 Generally, the experimental data related to SC
2 analyses appear discordant, and the differences in the
3 definition of the SC (as debated above in paragraph
4 2) strongly generate confusion and, as a consequence,
5 different manners of interpreting the data. Similarly,
6 the discrepancy of results concerning the SC is certainly
7 due to the lack of a fine method of detection,
8 able to appreciate slight differences in terms of size as
9 well as to monitor the quickly evolving and mutable
10 binding states of the SC. Moreover, it is interesting
11 to note that the major part of this investigation on
12 SC structure belongs to experiments on inorganic
13 NPs such as SPION, silica and gold NPs. The major
14 dispersion in size distribution of organic polymeric
15 NPs is probably one of the critical point that limits
16 PC studies on these particles; data relating their
17 *in vivo* behaviour are poor or totally lacking.
18 Researching a reliable method to discriminate the SC
19 contribution to the total PC structure is actually an
20 urgent issue, especially for organic polymer NPs.

21 22 23 **Binding Affinities and Stoichiometry of** 24 **Proteins in the SC**

25 The binding and dissociation rates of proteins to NPs
26 are surely critical parameters for their biological fate.
27 It is widely accepted that the tightly associated pro-
28 teins of the HC (with slow exchange rate) may follow
29 the particle during the endocytosis process, while
30 proteins of SC (with fast exchange rate) are quickly
31 replaced by the intracellular proteins, during or
32 immediately after endocytosis.⁸³ As a consequence,
33 the SC is generally considered less relevant in govern-
34 ing the functional response of NPs. However, the
35 biological outcome may differ if, not only endocytosis
36 process, but also the relative protein exchange
37 rates between NPs and cellular receptors, are consid-
38 ered. Because the protein–ligand complexes typically
39 display lifetimes from microseconds to days,¹⁷ it is
40 feasible that the fast exchanging proteins of the SC
41 could be strongly involved in determining the biolog-
42 ical fate of a NP, even if the rates of association and
43 dissociation are likely to vary quite considerably
44 depending on the protein and particle type.

45 As previously reported, methods are generally
46 oriented to compare the protein exchange rates of
47 the total PC; in this paragraph, we principally discuss
48 studies dealing with protein binding affinity in the
49 total corona, as the discrimination for SC proteins
50 could only be hypothesized from the total PC
51 analysis.

52 In this way, a mathematical dynamic model
53 was developed aiming to predict the time evolution
54 and equilibrium composition of the total PC based

57 on protein affinities, stoichiometries, and rate con-
58 stants. The authors applied both the theoretical
59 model and experiment procedures (by SPR technique)
60 to polymeric NPs (NIPAM/BAM) interacting with
61 three model proteins [HSA, high-density lipoprotein
62 (HDL) and fibrinogen]. Experiments indicated that
63 the PC evolves with time (as predicted by the model),
64 with evidence of HSA presence in the SC and HDL
65 presence in HC.⁸⁰ These findings nicely correlated
66 with the results previously described on the charac-
67 terization of the PC binding affinity, where HSA
68 showed low binding affinity (ascribable to the SC)
69 around hydroxyethyl starch nanocapsules.⁶¹

70 Moreover, the protein binding affinity study
71 can be helpful to describe how some NP features
72 could affect the protein exchange rate of the PC. In
73 an elegant study, Cedervall et al., using SEC and ITC
74 techniques, investigated the impact of different copol-
75 ymer ratios and different rates of hydrophilicity/
76 hydrophobicity of NIPAM-BAM NPs on the associa-
77 tion and dissociation of HSA and fibrinogen. Results
78 suggested that protein dissociation is affected by the
79 surface properties of NPs (exposition of functional
80 groups, hydrophilic/hydrophobic surface balance)
81 and in particular, dissociation was faster considering
82 the hydrophobic particles.¹⁷

83 However, most of the kinetic modeling of corona
84 complex formation is operated through *in silico* stud-
85 ies. Mathematical modeling helps to learn principles
86 and to develop quantitative approaches that cannot be
87 experimentally extracted. Moreover, mathematical
88 models provide quali/quantitative endpoints, useful for
89 the design and evaluation of experiments. In this view,
90 different approaches in the literature are proposed. For
91 example, Darabi Sahneh et al. presented a model to
92 describe two-phases of corona complex dynamics,
93 based on two formulae that predict corona composi-
94 tion of simulations through insertion of appropriate
95 parameters depending on features of the NPs.⁸⁴ The
96 authors assert that one potential application of this
97 model would involve a single cell culture medium
98 related to a complex protein medium, such as blood or
99 tissue fluid. On the contrary, Vilaseca et al. simulated
100 molecular dynamics to study the surface adsorption of
101 proteins. The authors reduced the complexity of a full
102 modeling by approximating protein molecules as sin-
103 gle, rigid entities. Kinetic modeling of the corona com-
104 plex formation process dramatically decreases
105 computational cost, though adopting several simplifying
106 assumptions. Finally, *in silico* analysis can be
107 applied to predict the final *in vivo* response of NP–PC
108 complexes.⁸⁵

109 *In silico* prediction analyses were also applied
110 to predict the evolution and subcellular distribution

of NPs in living cells^{84,86,87}; the interactions between NP–PC complexes and cellular membranes were investigated showing that the PC may enhance phagocytosis of positively charged NPs, but also cause the loss of targeting activity of both hydrophobic and positively charged NPs towards cancer cells.

Protein Conformation and Composition of the SC

As previously reported, the studies on protein conformation are all referred to the HC. In the SC, the proteins, loosely bonded to the NPs surface or displaying weak interaction with HC, are characterized by a fast dissociation rate, making the detection and full characterization of structural changes particularly difficult. Similarly, only a few papers describe the composition of the SC and generally, complex experimental procedures are required to recognize the proteins involved.

AOS Ashby et al. (2013) presented an alternative method to analyse the SC consisting of the flow field-flow fraction (F4) technique.¹⁸ Upon incubation with depleted serum (human serum without albumin or IgG), half of the NPs were centrifuged for coprecipitation of ‘all’ bound proteins. The other half was injected on the F4-column to remove proteins bound with fast exchange kinetics (SC), thus leaving only the proteins bound with slow exchange kinetics (HC) to be co-isolated with the SPIONs. The proteins collected with the SPIONs were digested and analyzed by two-dimensional PAGE and nano-LC–MS/MS for identification. Through mass identification of the total protein after NP–PC complex centrifugation and subtracting the protein identified on the surface of the NPs after F4-column elution, the authors supply a list of proteins characterizing the SC.

Surely, this method permits the discrimination of those proteins binding with fast exchanging kinetics, belonging to the SC, but on the other hand it appears debatable that ‘all’ the proteins forming the PC can be isolated by centrifugation. Confirming this lack of clearness, other authors assessed that centrifugation of the NP–PC complex inevitably leads to a perturbation of the system and, as a consequence, to the partial loss of the loosely binding proteins.^{17,88}

Alternatively, Sakulkhu et al. proposed a different approach regarding SPION-PC characterization. After the incubation of NPs in serum, SPIONs surrounded by the PC were entrapped into a magnetic column and the protein was eluted by means of various buffers with different ionic strengths, in order to separate the proteins from the NPs.¹⁴ In particular, to investigate the SC, the researchers applied a first

wash with PBS to separate loosely bound proteins, followed by washes with solutions of up to 2 M KCl. Finally, those proteins which remained bound to the NPs were called ‘tightly bound’ proteins. In this way, the final result is a triple partition of the total PC: SC, HC and ‘tightly bound’. Each elution fraction was analyzed by SDS–PAGE coupled to LC–MS/MS to protein identification. This technical strategy allowed for a fine characterization of the whole PC in general, but in particular, permitted the investigation of the SC composition. Indeed, the magnetic separation technique and the magnetic properties of SPIONs are useful to overcome problems of SC isolation in order to characterize its composition, but, on the other hand, this technique can inevitably only be appreciated for a few fields of application. The obtained results, reported in the article, showed that ‘tightly bound’ proteins were observed only on negatively charged PVA-coated SPIONs after the strong protein elution. The triple partition of total PC represents a novelty in this research field and, one more time, is proof of the great confusion about appropriately defining the PC. Nevertheless, the work of Sakulkhu et al. is well organized; no parameters exist to establish what are the HC, SC and ‘tightly bound’ proteins. Thus, the triple partition of the article results are arbitrary and non-comparable with other articles in which only a bi-partition of the total PC is present.⁸¹

Apart from SPIONs, the only characterization attempt for SC composition can be ascribed to Cedervall et al.¹⁷ Using SEC, researchers were able to distinguish both fast and slow components of the PC (as discussed above in relationship with binding affinity of SC proteins). Furthermore, they also were able to collect the proteins with fast exchange rate (ascribable to the SC) and characterize them through SDS–PAGE. In this manner, they compared the NP-associated protein received after centrifugation and after SEC isolation. In particular, through SEC protein isolation, they found that HSA and fibrinogen concentrations dominate on the particle surface. On the contrary, apolipoprotein A-I (a lower plasma abundant protein with higher affinity and slower kinetics ascribable to the HC) was the most abundant protein recovered after centrifugation.¹⁷

INSIDE THE METHODS: LIMITATIONS AND ADVANTAGES

Aiming to analyse the HC composition, the choice between ex-particle or on-particle approaches still remain an open issue. As confirmation of this

1 uncertainty, some authors described some defects
2 and limitation of the ex-particle approach, especially
3 if the proteins are only identified by 2D-PAGE after
4 the desorption step.⁵⁷ In their opinion, the ex-particle
5 approach can easily allow a sample contamination
6 by albumin (the most abundant protein in plasma).
7 Furthermore, 2D-PAGE bases protein identification
8 on the comparison of the respective spot positions
9 with a standard reference map. This methodology
10 may lead to misinterpretation of the data due to
11 spots overlapping, especially with complex pro-
12 teomes, like human plasma. Besides this limitation, it
13 is true that the ex-particle method allows better sam-
14 ple fractionation as well as multiple analyses
15 (i.e., electrophoresis tandem-mass spectroscopy) that
16 lead to a major number of recognized proteins.

17 So, the main drawback on HC analysis is not
18 regarding the kind of approach (on-particle or ex-
19 particle), but how to obtain a solid result independ-
20 ently from the chosen approach. In this way, a
21 multiple-technique protocol (i.e., electrophoresis +
22 mass spectroscopy, or chromatography + electropho-
23 resis) can represent a good solution.

24 Beside this aspect, the overlap of different tech-
25 niques of investigation and advances in instrument
26 technologies and software has allowed to reach an
27 earlier mapping of the HC of different NPs on the
28 basis of size and material composition. There are
29 review-tables in which it is possible to recognize dif-
30 ferent type of NPs and the related identified proteins
31 of the HC.⁸⁹ One of the most important aspects and
32 findings is the reproducibility of the data on compa-
33 rable NPs exposed to similar incubation condition
34 showing comparable results in terms of the HC
35 composition.

36 Regarding the SC analysis method, a shortage
37 of investigative methods and, as a consequence,
38 shortage of available data concerning the SC, does
39 not allow speculation on the weakness and strengths
40 of the analysis and of the resulting data. In particular
41 poor specificity, low reproducible rate of results and
42 poor applicability-range of some techniques designed
43 'ad hoc' for specific typologies of NPs represent the
44 most important limitations.

45 Finally, it is worth to mention that almost all
46 the studies concerning HC and SC have been carried
47 out *in vitro*. This is mainly due to the difficulty of
48 capturing NPs after administration. Nevertheless, the
49 importance to understand structure-activity relations
50 linking NPs and proteins adsorbed on their surface
51 to physiological responses is needed for effective bio-
52 medical application of NPs. Improving the ability to
53 predict the biological outcomes of NPs will speed up
54 their translation to the clinic. As a matter of facts,

the recognition of specific sequences of peptides
drives key biological processes, such as receptor-
mediated cellular association, particle retention in tis-
sues and organs, and ability (or inability) to cross
biological barriers. To date, we are still unable to
decipher the mechanisms regulating the interaction
between PC-covered NPs and biological systems and
more studies are needed. Deciphering the biological
recognition between PC proteins and cell receptors
could help us understand exactly how protein-
decorated NPs interact with cells and biological bar-
riers, potentially activating different biological
pathways.⁹⁰

CONCLUSION AND FUTURE PROSPECTIVE

The last 20 years of research in nanomedicines have
taught us that the composition of the nanoparticle
itself was the most important keystone impacting the
destiny of NPs. Nowadays, we must be aware that
nanoparticles are not only formed by 'polymers and
drugs', but are associated with proteins, stably or
weakly adsorbed onto their surface. Nanoparticles
and their PC are new 'biological entities'.⁹¹ These
interactions strongly impacts (maybe more than the
composition of the nanoparticles, size, and shapes)
their safety and functionality performances.

Since this concept is relatively new, a number
of issues are now up for debate:

1. Is the physiological response of a nanomedicine (meaning drug delivery system + associated PC) influenced by the whole PC or only a subset?
2. Are the protein belonging to the SC implicated in physiological response or not?
3. Are the technologies suitable and sufficient to discriminate and describe the HC, SC, or both?
4. What competences and skills are needed to completely understand the impact of the PC on the destiny of nanomedicines?

To-date, many of these questions are almost completely unsolved, but some indications and future direction could be hypothesized.

Firstly, it is reliable that the whole PC influences the biodistribution of PC-NPs complexes, but it could be hypothesized that some specific NP tropisms or accumulation could be due to a selective interaction of a subset of the associated PC with specific cells or receptors.

Moreover, the most debated aspect of the PC 'area' is the role of the SC and HC in determining biological effects. Some authors hypothesize that the SC is not pivotal in governing the biological destiny of nanomedicines, other authors are fully convinced of the contrary while some others researchers described the role of the SC of a minor importance with respect to the role of the HC.

A clear knowledge of these aspects is critical since it could strongly help in designing nanomaterials able to interact with proteins and cells in a controlled way.^{92,93} As a consequence of the lack of this knowledge, most nanomedicine are created specifically aiming to suppress protein adsorption. This

would reduce off-target cell uptake, but also lowers targeting efficiency.^{94,95}

Another important lack in PC research is connected with technologies. PC-NPs complexes could be characterized by integrating information on morphology (imaging-spectroscopy-scattering based techniques)⁴¹ and on structure/composition of the PC (cryo-electron microscopy and protein crystallography).¹⁵ Thus, multi-disciplinary approaches are needed in order to obtain much more information about the PC and its properties to fully understand the real impact of the PC on nanomedicines, and therefore to better support a more safety and conscious application of nanotechnology in medicine.

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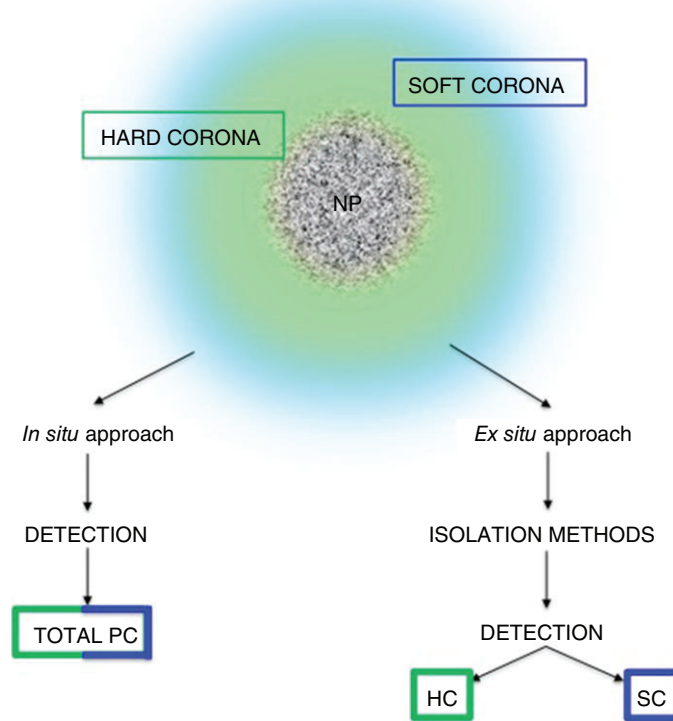
1 **Graphical abstract**

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3 **Protein corona and nanoparticles: how can we investigate on?**

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5 Francesca Pederzoli¹, Giovanni Tosi¹, Maria Angela Vandelli¹, Daniela Belletti¹, Flavio Forni¹, Barbara Ruozi¹

6
7 **AQ3**

8 METHODS FOR PROTEIN CORONA CHARACTERIZATION



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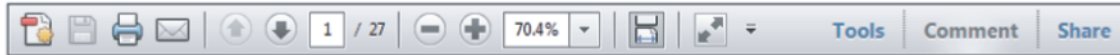
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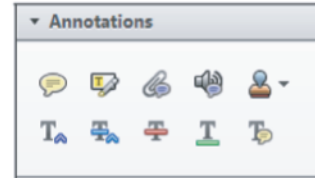
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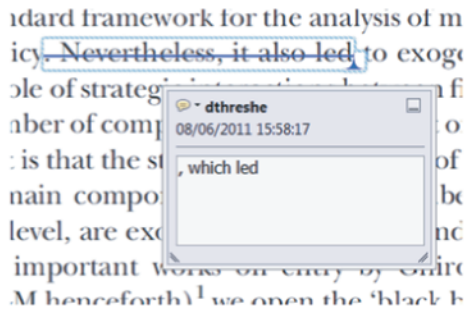


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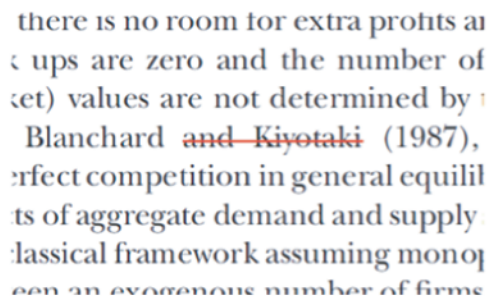


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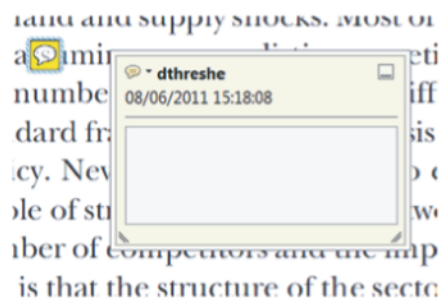


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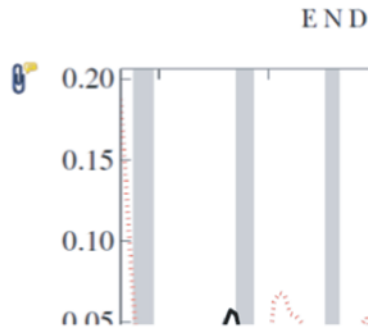
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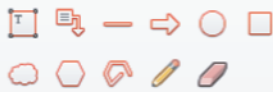
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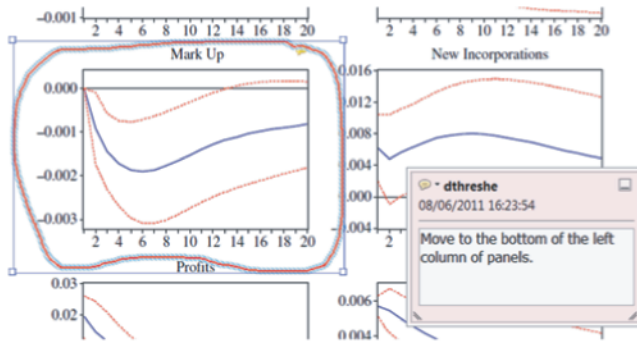


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