INTELBIOMAT project

Understanding the strength of the binding of the protein corona around nanoparticles.

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Purpose of the visit

When nanoparticles are dispersed in a biological medium they encounter high concentration of macromolecules that due to the high surface energy of the nanoparticles adsorb on these forming layer(s) of macromolecules (including proteins) that lead to the formation of what is described as "corona". This corona play an important role in the biodistribution of the nanoparticles inside living organism and if nanoparticle are used as a drug carrier could act as a restraint for the effective targeting of the nanoparticle to the desired organ.

It is well know that the protein corona around nanoparticle is made of a Soft or Dynamic corona forming an outer layer and in which the proteins are loosely bound and are in exchange with the surrounding solution and can be eventually replaced by other proteins, and an hard shell of proteins (the so called Hard Corona (HC)) that are strictly associated with the nanoparticle surface. The objective of my visit is to understand the stability of the binding of the proteins forming the plasma Hard Corona on silicon (SiO_2) and Carboxy modified Polystyrene (PS-COOH) nanoparticles and the relative exposure of their epitopes.

This has been pursued by performing a physicochemical characterization of the protein corona as a whole and stripping selected proteins (by means of appropriate surfactant solutions) while the exposure of the epitopes has been investigated by means of Differential Centrifugal Sedimentation (DCS), using an appropriate antibody for the detection of the selected proteins.

Description of the work carried out and main results obtained during the visit

During my visit at CBNI my work was focused on the evaluation of the stability of the binding of plasma proteins forming the so called Hard Corona (HC) on the surface of silicon oxide (SiO_2) and Carboxy modified polystyrene (PS-COOH) nanoparticles (Nps). In collaboration with my supervisor we decided to use these Nps since they are very well characterized by Mass Spectrometry from the point of view of the protein corona composition and in particular we used the same surface area/protein ratio previously described by Kenneth Dawson's group (we used 1 mg/ml concentration for the 100 nm SiO_2 particle and 0.42 mg/ml of concentration for the PS-COOH particle see for reference Pitek, A. S. et al *PloS one* **2012**, *7*, e40685., and Monopoli, M. P. et al. *JACS* **2011**, *133*, 2525–34.). Moreover these nanoparticle are easily dispersed even after their resuspension in solution containing human plasma proteins.

At this purpose we made protein corona by incubating Nps at 37°C for 1 hour in a Phosphate Buffer Solution (PBS) containing increasing concentration of human plasma proteins (HP). The resulting Nps-corona complexes were separated from the excess of plasma by centrifugation and extensive washing as previously described (see Monopoli et al., 2011 JACS). The proteins adsorbed on the Nps were then eluted using standard protocol (Monopoli et al, 2011 JACS) and separated by SDS PAGE (Sodium Dodecyl Sulphate Poly Acryl Gel Electrophoresys). After that the gel was Comassie stained following procedure previously described (Bradford et al, 1974 Analytical Biochemistry). Figure 1 A and B show the SDS PAGE of the proteins adsorbed on SiO_2 and PS-COOH particles incubated with increasing concentration of human plasma solution. As the figures show there is a change of the pattern of the proteins adsorbed on the surface of the particles increasing the plasma protein concentration. This is probably due to a competitive binding of the proteins with the Nps surface.

We complemented the structural data of the Hard Corona on these two materials with DCS measurements. DCS measures particles size distribution using centrifugal sedimentation within an optically clear spinning disc. In particular in this case the particle-protein complexes were injected in a sucrose gradient pre-

equilibrated in the instrument. The graph in Figure 1-C shows that for SiO_2 particle there is a decrease of the actual size after the incubation with human plasma. This effect can be overcame, if we normalized with a core shell model that take in account for the different density of the two materials (the protein shell and the Np core):

$$\frac{(\rho_c - \rho_s)}{(\rho_c - \rho_f)} \frac{{D_c}^3}{D_s} + \frac{(\rho_s - \rho_f)}{(\rho_c - \rho_f)} {D_s}^2 = D^2$$

Where ρ_c is an inner core of density, ρ_s is shell of density, ρ_f is fluid of density, D_c is diameter of the core, $D_s(D_c+2\delta)$ is the total diameter of the core-shell particles, and D is a measured diameter by DCS method.
We did not observed this effect with polystyrene Nps, since polystyrene has a density very similar to that of proteins.
A deeper analysis of the DCS measurements highlight the formation of multimer for the PS-COOH Nps in low plasma concentration while the complexes are mainly monodispersed in high plasma concentration, with the formation of a second small population of dimer.
Interestingly the SDS-PAGE data are in agree with DCS measurements since the changes in protein composition are mirrored at a structural level by the changes in particle-protein size. This means that on both materials the competitive binding drives a decrease of the protein corona thickness increasing the plasma concentration underlying the different behaviour of the nanoparticle using in vivo (high HP) or in vitro condition (low HP).
In order to clarify the mechanism and the stability of two compositionally different HC we decided to use different protein concentrations using a low plasma concentration (10% Plasma) and high plasma concentration (55% Plasma),
For doing this we used a strategy that exploits the desorption of the proteins forming the corona on the Nps surface using different surfactants. In particular we carried out the study using a zwitterionic, a non-ionic, an anionic and a cationic surfactant. We used two surfactant concentration, one below the CMC (critical micellar Concentration) (0.01% w/v), and one above the CMC (1% w/v). This provide an example of a controlled systematic variation of properties that could affect the protein corona composition and can allow to infer on the mechanism of its formation, the organization and the strength of the binding of the protein on the nanoparticle surface.
In particular Hard Corona has been incubated with a PBS solution containing the selected surfactant for 1 hour at 37°C. After that the fraction containing the protein still bound to nanoparticle surface (RF) has been separated from the supernatant containing the desorbed proteins (DF). All the data reported are relative to the RF fraction. In the following results I will show SDS PAGE reporting in the first lane a molecular weight marker, in the second line the Hard Corona protein composition, in the third and the fourth line the proteins presents in the Hard Corona after the treatment with a PBS solution containing respectively 0.01% and 1% of the selected surfactant.

We found that HC made in high plasma protein concentration is more stable than the one made in low plasma protein concentration, since in the second case the proteins are resistant to the desorption with mild detergent like TRITON-X100.
Moreover, we found that it is possible to selectively desorb one single protein from HC on $SiO2_2$ particle made with 55% plasma solution by resuspending Np-HC complexes in a PBS buffer solution containing 1% of the mild zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propansulfonate (CHAPS).

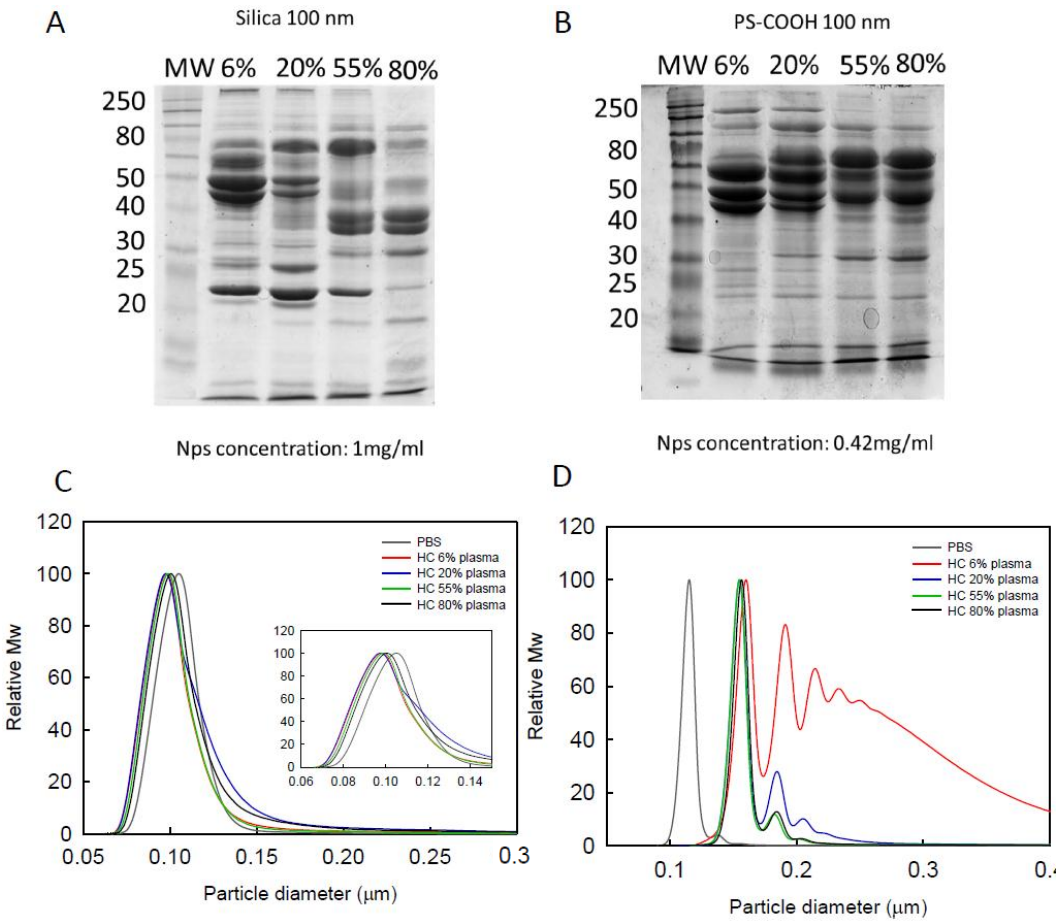

Figure 1. SDS-PAGE gel of human plasma proteins eluted from **(A)** 100nm SiO_2NP-protein complexes or **(B)** 100nm PS-COOH NP-protein complexes free from excess plasma and obtained using different plasma concentrations. The molecular weights of the proteins of the standard ladder are reported on the left for reference. DCS measurements of 100 nmSiO_2 particle-corona complexes **(C)** or 100 nm PS-COOH **(D)** particle-corona complexes free from excess plasma and diluted in PBS. The particle-corona complexes were isolated after 1 h of incubation in plasma solution at different concentrations, as shown in the legend.

Figure 2 shows Hard Corona data obtained after the desorption of the proteins from SiO_2 Nps previously incubated with 10% human Plasma solution. In particular in the panel A is reported an SDS PAGE showing the proteins present in the RF after the desorption with the zwitterionic detergent CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. Based on MS data of previous paper (Monopoli et al JACS 2011) it seems that this surfactant is able to desorb mainly the apolipoproteins from the nanoparticle surface. The desorption of the proteins is linked to the formation of the micelles since just high surfactant concentration (line 4 of the SDS PAGE) is effective in the desorption.

In panel B we show the proteins of the Hard Corona after the desorption with TRITONX-100 (TRITON) a nonionic surfactant characterized by an hydrophilic polyethylene oxide group and a hydrophobic group. The hydrocarbon group is a 4-(1,1,3,3-tetramethylbutyl)-phenyl group. Even In this case the desorption of the proteins is mainly driven by the formation of the micelles while for low concentration of the surfactant few proteins are desorbed. Interestingly Histidine Rich Glycoprotein seems to be the most strongly bound protein to the nanoparticle surface. The differences between the two effect can be found in the different ability to lower the surface tension of the surfactants.

The addition of SDS (Sodium Dodecyl Sulphate) a strong denaturing surfactant seems to be able to desorb most of the proteins of the HC (Figure 2-C). In this case for concentration below the CMC (0.01% SDS) we

have the desorption of the apolipoproteins, while for concentration above the CMC (1% SDS) most of the proteins present on the Nps surface were desorbed.

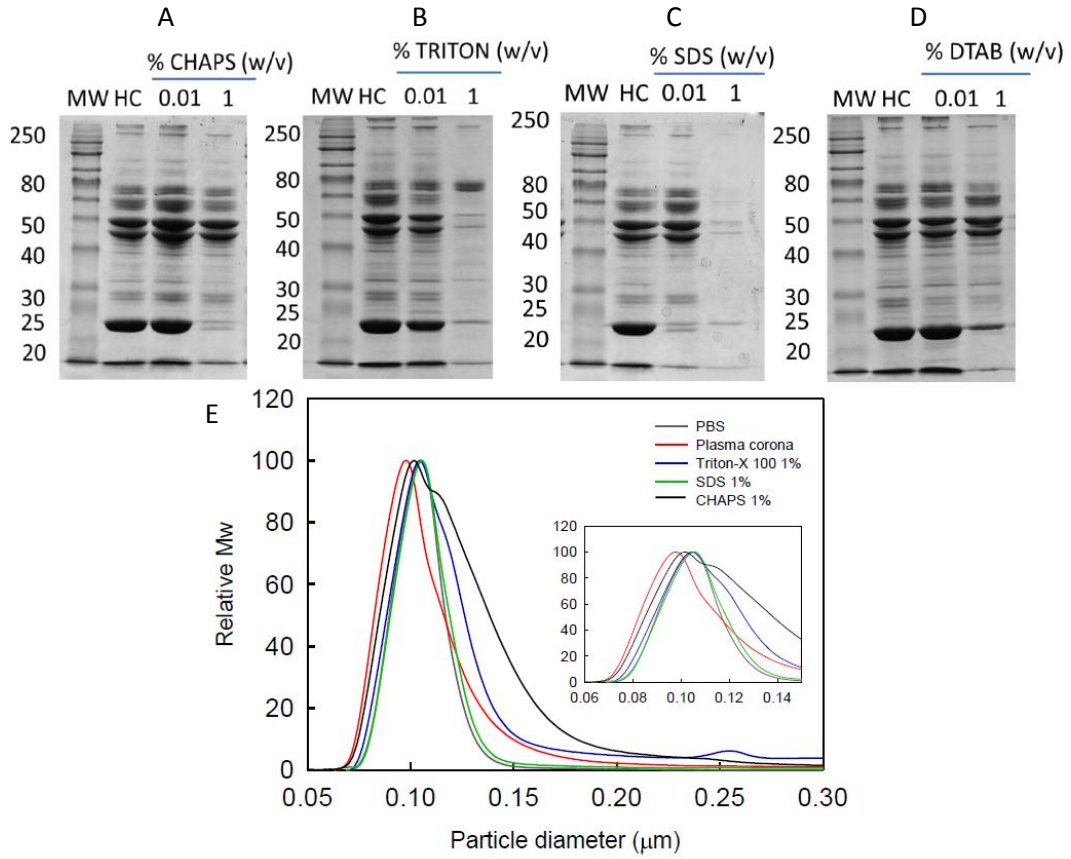

Figure 2. SDS-PAGE gel of human plasma proteins obtained from 100nm SiO_2 incubated with a PBS solution containing 10% Human Plasma. NP-protein corona complexes were afterward incubated for 1 hour at 37°C in a solution containing increasing concentration of **(A)** CHAPS, **(B)** TRITON, **(C)** SDS or **(D)** DTAB. **(E)** DCS experiments with the nanoparticle corona complexes obtained incubating Nps in a PBS solution containing 10% Human Plasma. Nanoparticle corona complexes free from excess of plasma were afterwards incubated for 1 hour with a PBS solution containing 1% (w/v) of the selected surfactant, as shown in the legend.

Finally we investigated the resistance of the HC in the presence of the cationic surfactant DTAB (Figure 2-D) . In this case low amount of proteins were desorbed even with high concentration of surfactant. This is probably due to the stabilization effect that DTAB exert on the binding of protein to silicon oxide surface.
Panel E shows the distribution of nanoparticle-protein complexes after the resuspension in the buffer solution containing the selected surfactant as measured by DCS. The desorption of the proteins can be followed monitoring the decrease in the shell thickness of the protein corona. Interestingly the decrease in the size is in agree with the amount of protein desorbed. In particular the graph shows that HC has one main peak and the complex are relatively monodispersed while the desorption of the proteins with CHAPS or TRITON leads to the agglomeration of the complexes. Interestingly the addition of the SDS reduces the size of the complexes to that of the bare particles while the addition of DTAB leads to particles aggregation probably neutralizing the negative charge of the silicon oxide particle.
Summarizing we found that 10% protein corona is not very stable since even after the desorption of few proteins we observed the formation of Np-protein complexes that are not completely monodispersed. This is

well in agree with data present in literature that show that the proteins itself are the main force that allow the formation of monomeric Nps - protein complexes.
The study on SiO2 surface is complemented by data obtained for high plasma proteins concentration (55% human plasma). In this case we found that the hard corona is very stable, since it resist to desorption with both CHAPS and TrironX-100. In particular Figure 3 A-B show the protein pattern for the RF fraction of the HC treated with CHAPS or TRITON. We found that both the surfactants drive mainly the desorption of just one protein that is the Apolipoprotein AI (ApoAI), the protein highlight in red Figure 3 A-B.

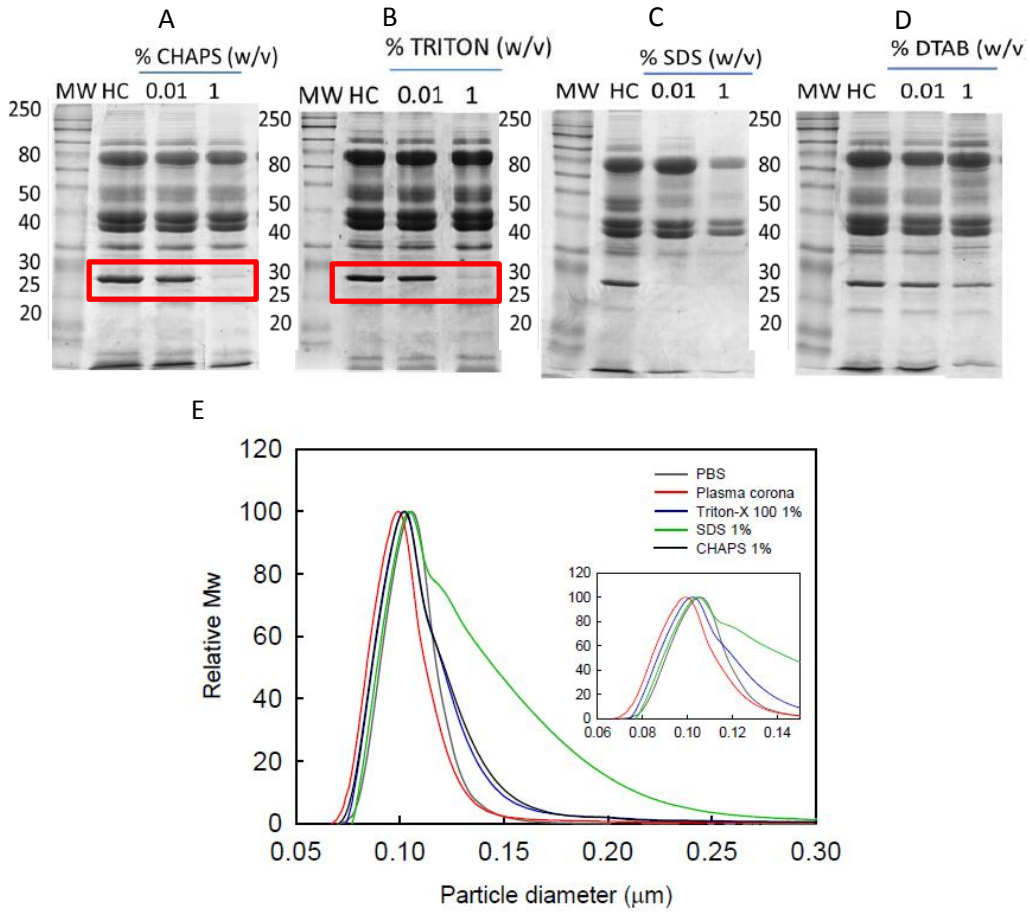

Figure 3. SDS-PAGE gel of human plasma proteins obtained from 100nm SiO_2 incubated with a PBS solution containing 55% Human Plasma. NP-protein corona complexes were afterwards incubated for 1 hour at 37°C in a solution containing increasing concentration of **(A)** CHAPS, **(B)** TRITON, **(C)** SDS or **(D)** DTAB. **(E)** DCS experiments with the nanoparticle corona complexes obtained incubating Nps in a PBS solution containing 55% Human Plasma. Nanoparticle corona complexes free from excess of plasma were incubated for 1 hour with a PBS solution containing 1% (w/v) of the selected surfactant, as shown in the legend.

We performed semi quantitative Mass Spectrometry analysis for the estimation of the yield of the desorption of ApoAI and we found that treatment with 1% CHAPS is able to remove 92% of the proteins from the HC. Even in this case the desorption is driven just for concentration above the CMC.
The desorption of the proteins from HC on silica particles with SDS left on the Nps surface mainly two proteins that are HRG and IgG and are probably the ones with the highest affinity for the SiO_2 surface (Figure 3-C). Finally as in the case of the 10% HC, the incubation of the HC with DTAB seems to be not effective in the desorption of the protein from the Nps surface (Figure 3-D).
Even in this case SDS PAGE data are in agreement with DCS measurements that show a reduction of the actual size of the protein corona as soon as we change the surfactant and in particular we have a lower

reduction of the shell thickness using mild surfactant (CHAPS or TRITON) with respect to the strong one (SDS).
Overall the data presented for SiO_2 Nps show that corona made with high plasma concentration (55% human plasma) is made of proteins that are very strongly bound to Nps surface since they are not desorbed by mild detergent. On the contrary 10% plasma HC is made of protein easily desorbed and loosely bound Nps surface.

In order to have an overview of two compositionally different Nps we also studied the stability of the Hard Corona on carboxy modified nanoparticle. Even in this case we used two different plasma concentration: a low (10%) and an high (55%) plasma concentration.
In the case of the 10% HC we found that both CHAPS and TRITON are able to desorb most of the proteins from the nanoparticle surface. In particular as shown by SDS PAGE presented in Figure 4-A proteins are desorbed just for concentration of CHAPS above the CMC (1% CHAPS). On the contrary Figure 4-B shows that for TRITON even concentration below the CMC are effective in driving proteins desorption.
After that we investigated the resistance to the desorption in the presence of strong surfactant. Figure 4-C shows that SDS is able to desorb most of the proteins adsorbed on the nanoparticle surface. The desorption is driven just for concentration of surfactant above the CMC.
In the same way DTAB is able to drive proteins desorption even for concentration below the CMC. So it possible that, in a different way with respect to SiO_2 particles, even the surfactant monomer is able to destabilize the binding of the proteins with the nanoparticle surface.
DCS measurements of the particle-HC complexes show that as for the silica the addition of the different surfactants drives the release of different amount of proteins that lead to the reduction of the thickness of the proteins shell. In this case CHAPS seems to be the milder detergent while SDS is the stronger since desorbs most of the proteins from the Nps surface. Even in this case the addition of DTAB drives the agglomeration of the Nps and act by neutralize the negative charge of the COOH groups.
Finally we investigated the resistance to the desorption of the protein corona made dispersing PS-COOH Nps in 55% human plasma solution. Both CHAPS and TRITON are able to desorb most of the proteins from the Nps surface. In particular Figure 5-A shows the proteins eluted form PS-COOH Nps after the treatment of the nanoparticle protein complexes with increasing concentration of CHAPS. In this case just few proteins were desorbed even after the addition of concentration of CHAPS above the CMC while in the case of TRITON treated sample, even concentration below the CMC are effective in the desorption of the proteins from the nanoparticle surface (Figure 5-B). After that we investigated the resistance to the desorption in the presence of strong surfactant. Figure 5-C shows that SDS is able to desorb most of the proteins adsorbed on the nanoparticles surface but just using concentration of surfactant that are well above the CMC, while if we use low concentration tiny amount of proteins are desorbed. DTAB (Figure 5-D) is able to drive proteins desorption even for concentration below the CMC. Even in this case very low amount of protein are present in the RF fraction, and are mainly represented by HRG and Albumin.
As for SiO_2 the addition of the different surfactants drives the release of different amount of proteins that lead to the reduction of the thickness of the protein shell of the Hard Corona (DCS measurements in Figure 5-E). The reduction of the protein thickness is intertwined with the strength of the surfactant since CHAPS drives the desorption of less protein with respect to SDS. Even in this case the addition of DTAB leads the agglomeration of the Nps-protein complexes.

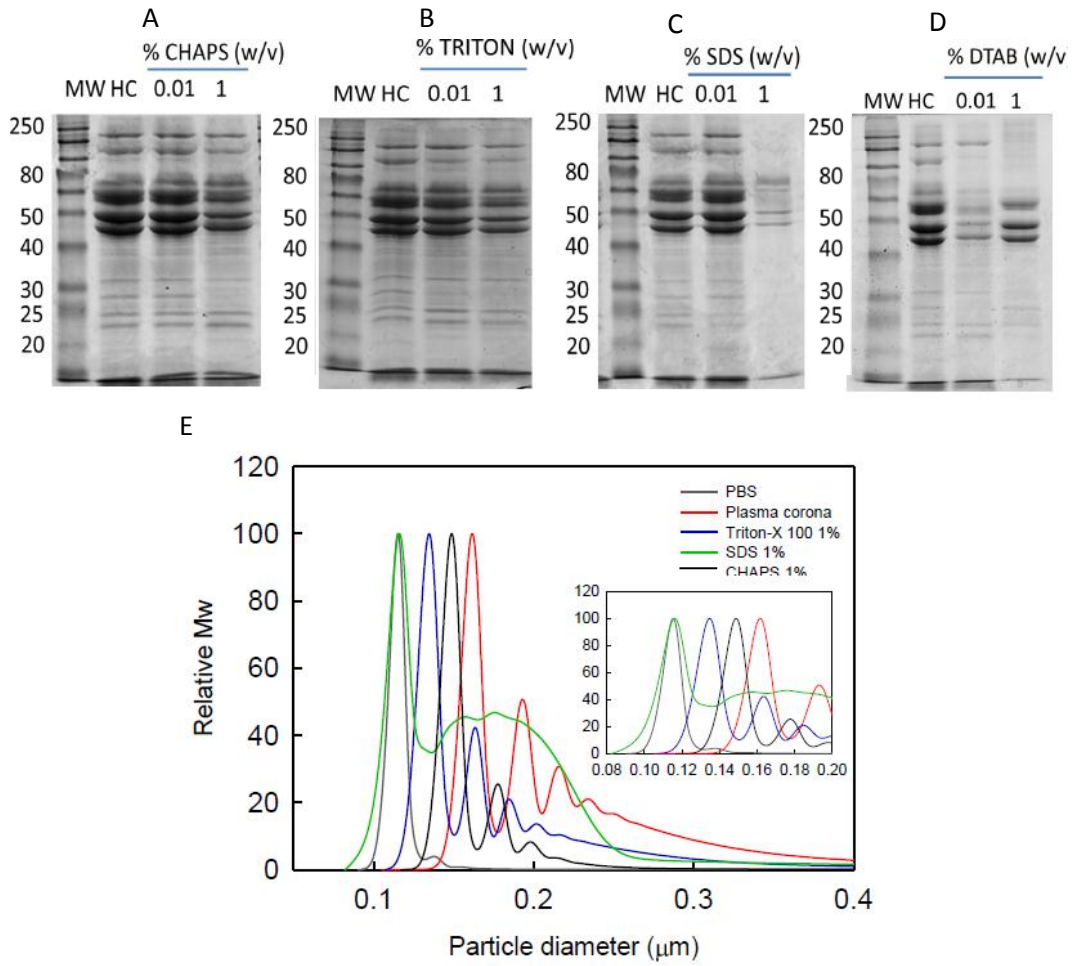

Figure 4. SDS-PAGE gel of human plasma proteins obtained from 100nm PS-COOH incubated with a PBS solution containing 10% Human Plasma. NP-protein corona complexes were afterwards incubated for 1 hour at 37°C in a solution containing increasing concentration of **(A)** CHAPS, **(B)** TRITON, **(C)** SDS or **(D)** DTAB. **(E)** DCS experiments of the nanoparticle corona complexes obtained incubating Nps in a PBS solution containing 10% Human Plasma. Nanoparticle corona complexes free from excess of plasma were afterwards incubated for 1 hour with a PBS solution containing 1% (w/v) of the selected surfactant, as shown in the legend.

Overall our results point to a change in the strength of the binding of the proteins with the nanoparticle surface changing the plasma concentration, since 55% plasma Hard Corona is "harder" that 10% plasma Hard Corona for both SiO_2 and PS-COOH Nps.

Moreover the case of the 55% Hard Corona on SiO_2 is very interesting since the addition of mild surfactants (like CHAPS or TRITON) leads to the desorption of one single protein from the nanoparticle surface. This could represent a good model for the study of the adsorption of a protein by Nps.
Since this we decided to investigate the kinetic and the thermodynamic of the binding of ApoAI with the SiO_2 surface.
In order to correlate the release of ApoAI from the Hard Corona with the thickness of the corona itself we monitored by DCS the decrease of the shell thickness after the addition of CHAPS.
At this purpose the Nps-Hard Corona complexes were incubated at 37 °C with a PBS solution containing CHAPS at a concentration of 1% (weight/volume) for different time. After that the nanoparticle protein corona complexes were directly injected in the sucrose gradient (Figure 6-A). Interestingly the release of the protein start after 10 minutes of incubation with CHAPS and then the desorption reaches the plateau after 60 minutes of incubation.

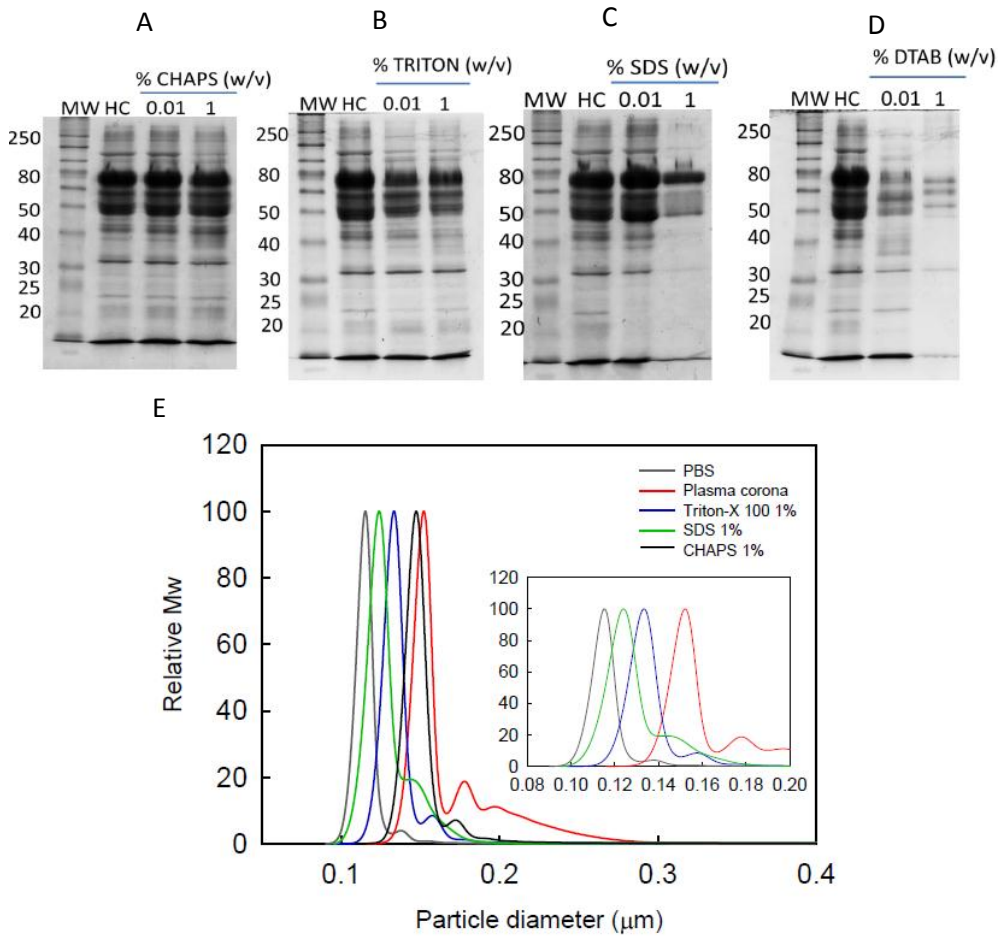

Figure 5. SDS-PAGE gel of human plasma proteins obtained from 100nm PS-COOH incubated with a PBS solution containing 55% Human Plasma. NP-protein corona complexes were incubated for 1 hour at 37°C in a solution containing increasing concentration of **(A)** CHAPS, **(B)** TRITON, **(C)** SDS or **(D)** DTAB. **(E)** DCS experiments with the nanoparticle corona complexes obtained incubating Nps in a PBS solution containing 55% Human Plasma. Nanoparticle corona complexes free from excess of plasma were afterwards incubated for 1 hour with a PBS solution containing 1% (w/v) of the selected surfactant, as shown in the legend.

The half time of the release of the protein from the hard corona in the presence of CHAPS is of 30.1±6.5 min (Figure 6 A-B). The binding of ApoAI to the nanoparticle surface was also characterized by Surface Plasmon Resonance. At this purpose Nps were immobilized to the gold surface with the linker molecule 3-MPTS ((3-mercaptopropyl) trimethoxysilane) and the binding of ApoAI and IgG, was tracked, at different concentration, during the time in order to evaluate the thermodynamic and kinetic constant for the interactions. The equilibrium response at each concentration were used in order to build a dose response curve.

The investigation of the binding of the proteins with the nanoparticle surface shows that ApoAI has a dissociation constant with Kd = 30 ± 1.4 nM while IgG another protein that bind to the nanoparticle surface with high affinity is one order of magnitude higher and the dissociation constant is Kd = 3 ± 2.5 nM . Both the kinetic constant kon and koff for the interaction between ApoAI and nanoparticle surface are shown in Figure 6-C. Interestingly the proteins have different physicochemical characteristic, since the pI of ApoAI is 5.2, whereas the pI of IgG is 8. Thus the proteins have different charge when they are dispersed in physiologic condition and in particular ApoAI is positively charged while IgG is negatively charged.

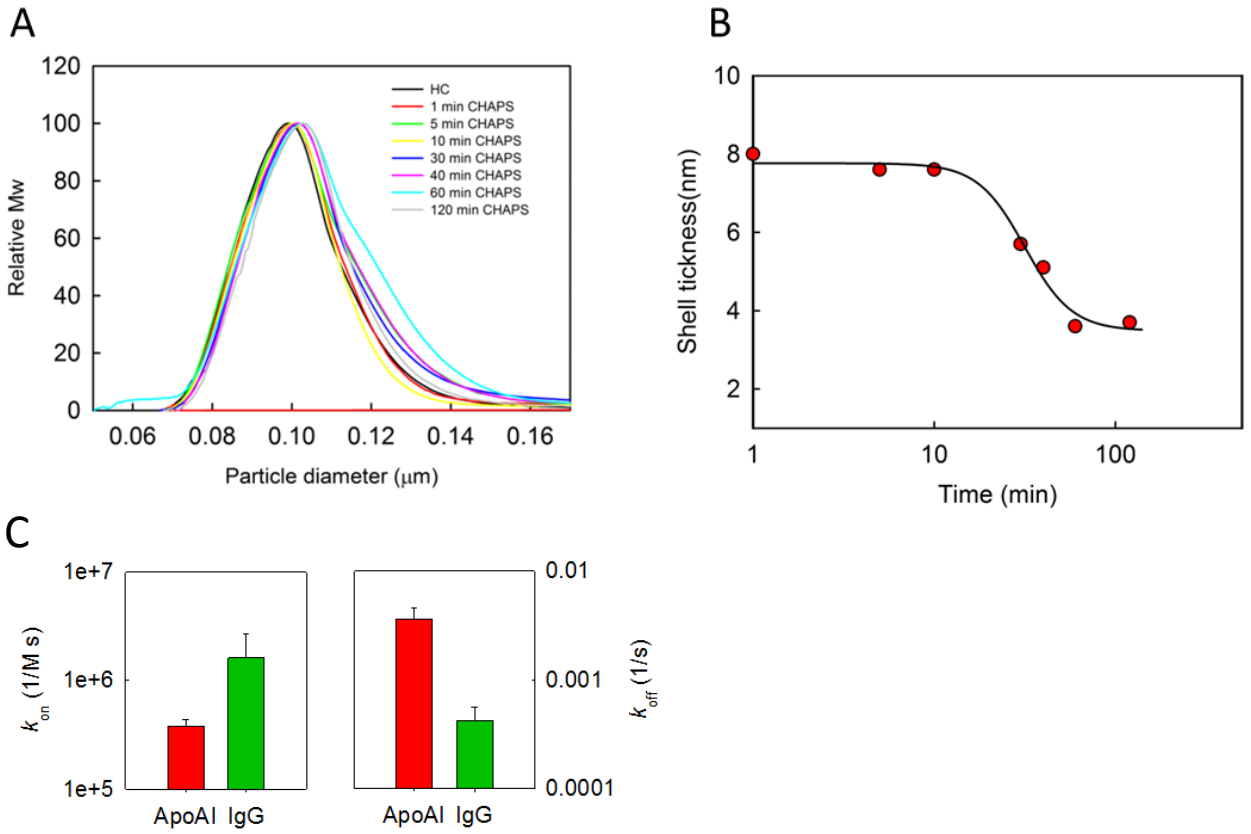

Figure 6 **(A)** DCS experiments with the nanoparticle corona complexes free from excess of plasma incubated for different time with 1% CHAPS. **(B)** Determination of the shell thickness of the Hard Corona after the treatment for different time with a PBS buffer solution containing 1% CHAPS using a core shell model and expressed as a function of time incubation. **(C)** Kinetic parameters for the interaction between silica Nps and ApoAI and IgG determined by SPR.

We complemented the structural data for the Hard Corona investigating the exposure of the epitopes of the main proteins forming the corona around silica and PS-COOH Nps using three different polyclonal antibodies able to recognize, Fibrinogen (Fib), Apolipoprotein AI (ApoAI), and Transferrin (Tf). I used as a control an anti mouse secondary antibody that is not able to recognizes human protein.

Interestingly the incubation of the antibodies with the Np-HC complexes drives a massive agglomeration that can be detected by DCS. The agglomeration is due to the divalent nature of the antibody that can act as a bridge, crosslinking different Np-protein complexes.

We investigated the expression of the different proteins on the HC of both SiO_2 and PS-COOH Nps that were prior incubated with different concentration of Human Plasma (10%, 55% e 80%).

Figure 7 A-B-C show the results obtained with SiO_2 particles. We found that the proteins have different exposure on the HC and in particular it seems that ApoAI is expressed in all the conditions tested.

On the contrary the expression of the fibrinogen is very high on HC made with low plasma concentration (10% human plasma) while its expression is reduced when we incubated with high plasma concentration (80%).

Interestingly Tf seems to be expressed just on HC made with 80% human plasma.

As a control of the experiment we used an irrelevant antibody that is not able to recognizes protein in all the condition tested.

In the case of the PS-COOH Nps (Figure 7 D-E-F) we found that ApoAI is expressed at low level on the surface of the HC and the expression does not change in the different condition.
Fibrinogen is expressed just on HC made with low plasma concentration, since if we analyzed 55% or 80% we can see a reduction of the agglomeration of the nanoparticle due to the decrease of the recognition.
Tf is not expressed in all the condition tested, so it could be that TF is expressed in an inner layer of the Hard Corona on PS-COOH Nps, since we detect Tf by MS.

Conclusion

- It is possible to investigate the strength of the binding of the proteins forming the Hard Coron exploiting surfactant mediated desorption.

- Different Hard Corona made with different plasma concentration have different binding strength for the Nps surface.

- Different Hard Corona have different surface exposure of the proteins.

The main results presented in this report will be part of a joint publication with the members of the CBNI. In addition the work that has been carried out during my visit at CBNI will be included in my PhD thesis and I also expected that the main results will be presented in International meetings. Nevertheless the project has allow to put the basis for future collaboration between the members of the CBNI and the University of Brescia.

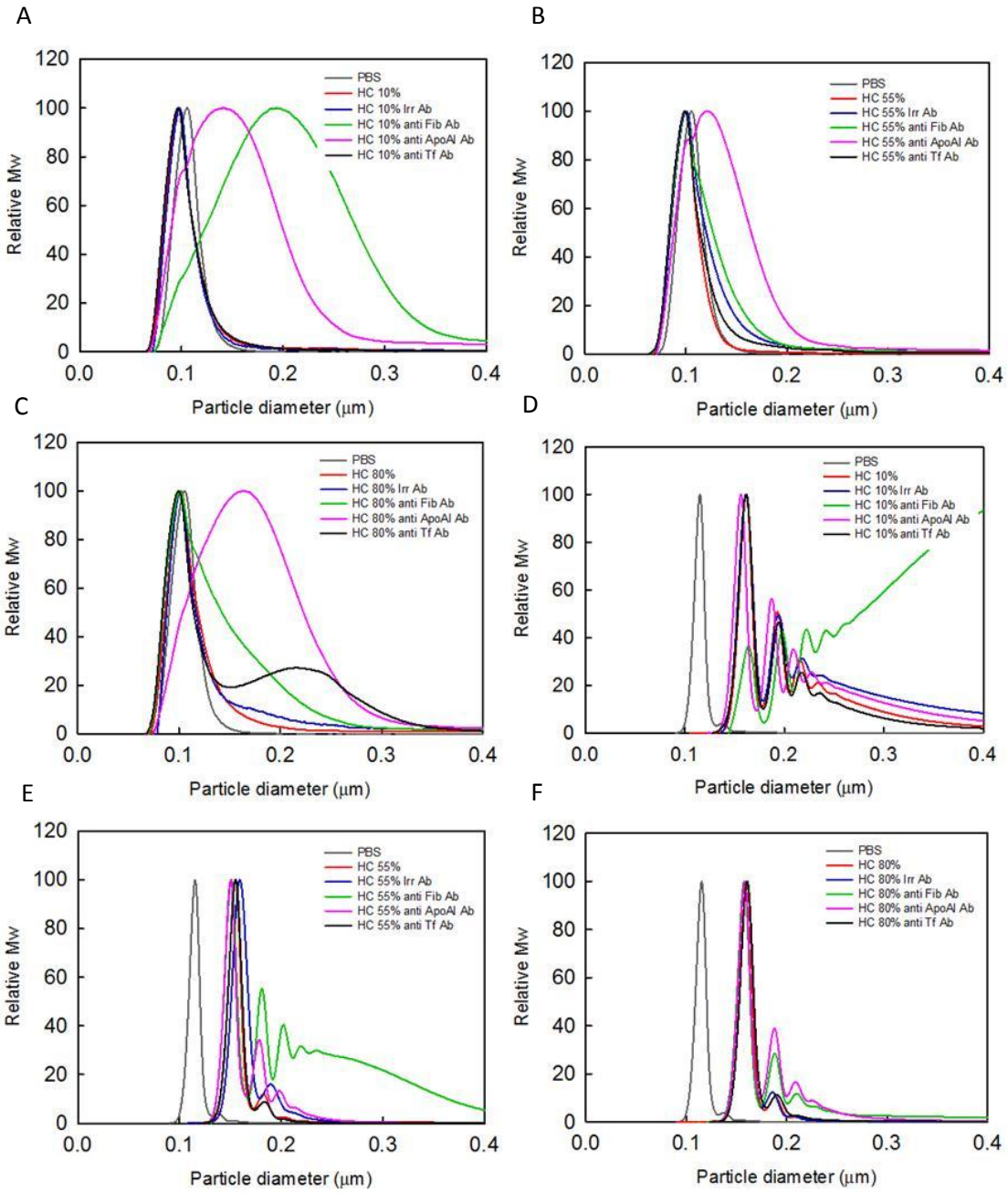

Figure 7. DCS experiments with the nanoparticle corona complexes free from excess of plasma incubated for 1 hour with different polyclonal antibody as shown in the legend. Different plasma concentration and materials have been used and in particular silica Nps were incubated with a PBS solution containing **(A)**10% human plasma proteins, **(B)** 55% human plasma proteins, **(C)** 80% human plasma proteins, or PS-COOH nanoparticle were incubated with a PBS solution containing **(D)** 10% human plasma proteins, **(E)** 55% human plasma proteins, **(F)** 80% human plasma proteins.