Report Epitope map short visit at CBNI, Dublin 24.7.11 – 4.8.11

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1. Introduction

In this work we are studying different protein corona molecules that are of microbiological origin. Microbial coronals can be acquired by nanoparticles through environmental routes and can significantly alter surface properties of nanoparticles. One especially interesting group of proteins in this context is the hydrophobins. They are produced in large amounts by filamentous fungi in the environment and are used by fungi to form surface coatings for adhesion and surface modifications. Hydrophobins have diverse roles in the growth and development of fungi, for example in adhesion to substrates, for reducing surface tension to allow aerial growth and in forming protective coatings on spores and other structures. The work performed here concerns the adsorption of hydrophobins on various polystyrene based nanoparticles. The ability of hydrophobins to adsorb on hydrophobic nanoparticles is extensively examined. Furthermore, we have recently shown that the hydrophobins HFBI, HFBII and HFBIII can increase the hydrophobicity of polar surfaces significantly. As hydrophobins have a role in the hydrophobicity of spores and hyphae, the following step taken is to examine the adsorption of hydrophobins on spherical, polar nanoparticles to replicate this attribute. Hydrophobins have also been shown to prevent immune recognition of airborne fungal spores giving hydrophobin coated nanoparticles application possibilities in medical purposes. The experiments used in the short visit includes NP production and following coverage and purification using e.g. dialysis, as well as methods such as dynamic light scattering, analytical ultracentrifugation, z-potential measurements and SDS-PAGE.

The proteins HFBI and HFBII were allowed to adsorb on three kinds of polystyrene (PS) (d=100 nm) NPs followed by a dialysis step for purification. Plain Polystyrene, carboxylated PS as well as Polyethyleneimine (PEI) modified PS Nps were used. After covering and purification via dialysis, DLS, Z-potential measurements and DCS experiments (Analytical ultracentrifugation) were performed, as well as human plasma corona measurements on covered and uncovered particles. Also, SDS-PAGE experiments on HFB or HFB + human plasma corona covered particles were carried out.

Here, HFBI and HFBII were allowed to adsorb on the NPs at pH 5,6 (10 mM PBS) around the pI of the proteins. Also, HFBII was adsorbed on PEI PS NPs at pH 8,9 (10 mM glycine-NaOH). Dialysis was carried out at pH 7,4 10 mM PBS for 5 days with buffer change every day. During the final day of dialysis, the PBS buffer was exchanged for MQ water to avoid aggregation of particles.

2. *Materials and Metods*

Nanoparticles:

Plain: Sulfonated polystyrene 100 nm 26mg/ml yellow green. (Polyscience)

Carboxylated: Carboxylated polystyrene 100 nm 20 mg/ml Fluorospheres (Invitrogen)

PEI: Polystyrene NPs modified with polyethyleneimine (PEI) 100 nm, 2,2 mg/ml CBNI modified.

Buffers

For protein adsorption onto NPs, PBS 10 mM pH 5,6 is used. (For hydrophobin adsorption on PS PEI NPs, 10 mM glycine-NaOH is used). In dialysis experiments, 10 mM PBS pH 7,4 is used.

Hydrophobin adsorption on PS NPs:

Final Np concentration: 1mg/ml. 1,5 mg HFB/ml equals a 5 fold coverage and 2,5 mg/ml equals a 8 fold coverage.

NPs and hydrophobins are dissolved in buffer and sonicated separately before adsorption. NPs are sonicated in mild sonication for 3 minutes and HFB a short mild sonication of about 10 seconds. 500 ul of 2 mg/ml NP solution is mixed with 500 ul of 3 or 5 mg/ml hydrophobin solution depending on level of coverage. I ml NP/HFB solution is put in a lab shaker at 500 rpm in 25° for 2 h for adsorption to be completed.

Dialysis

Float-A-lyzer G2, Spectrum Labs, USA, is used for dialysis in order to remove any free or loosely bound hydrophobin from solution. Before use, the dialysis device is dispensed in 1 l of MQ water for 30 minutes. 1 ml of Np/HFB solution is loaded into a washed Float-A-lyzer and which is put floating in 1 L of buffer under mild stirring for 5 days in +4 °C. In these experiments, PBS 10 mM pH 7,4 is used. The buffer is exchanged after 5 hours and then once each day. In order to avoid aggregation of covered NPs, the buffer is exchanged to MQ water during the last 24 hours and the samples are retrieved.

DLS and Z-potential

For DLS, dynamic light scattering, and Z-potential measurements, the Setasizer ZS, Malvern Instruments is used. Samples are taken from the dialysis device and diluted from 1mg/ml NP to 0,1 mg/ml in MQ water. Before the final step of MQ dialysis, a sample was taken for each NP-HFNB combination and dissolved similarly in PBS pH 7,4 and to be compared with the final water diluted sample for aggregation purposes. Particle size is measured in in 4 repeated runs in a disposable cuvette. Z-potential is measured in a Z-potential cuvette in 4 repeated runs.

Differential Centrifugal Sedimentation (DCS)

AS DSC instrument, DCS instruments is used. Samples from dialysis are diluted to 0,1 mg/ml NP and dissolved in MQ. For aggregation comparison, samples pre MQ dialysis are taken and dissolved in PBS pH 7,4. 100 ul sample is injected per measurement, 120 ul sample is loaded into syringe.

Protein corona

Human plasma is diluted to 10 % in PBS pH 7,4. NP at 1 mg/ml, coated or uncoated with hydrophobin, is used and diluted together with plasma and PBS; 1:1:8 for 10 % plasma; NPs 1 mg/ml; buffer, total of 1 ml. Mixture is incubated at 37° for 60 minutes in eppendorf LoBind tubes. After incubation, samples are washed via centrifugation in 20000 rpm in 50 min 3 repeated times where the supernatant is removed between each step. After the first centrifugation, 500 ul buffer is added between each step.

SDS PAGE

15 % 1D SDS PAGE gel is made according to protocol. 20 ul dialysis sample is mixed with 10 ul loading mix (Loading mix: dithiothreitol: SDS 3X loading buffer 1:10). Samples are heated at 100 degrees for 3 minutes. 12 ul sample I loaded into each well and allowed to run for about 50 min at 130 V. Silver staining is used as staining method.

Spin filtration

Experiments with spin filtration (Vivaspin 500, 100 kDa, GE Healthcare) were also carried out in order to test a possible substitute for dialysis. Aggregation was however shown to be a limiting factor in the use of this technique and the method was discharged after the first preliminary runs. Results from soin filtration will not be presented any more thoroughly herein.

3. Results:

DCS

The DCS output data for carboxylated PS NPs is seen in Figure 1. Pristine particles dissolved in MQ water 0,1 mg/ml is showed as a reference. For HFBI and HFBII an equal increase in particle diameter after adsorption can be seen as the peaks for the proteins shift to the right. This shift is in the area of 5 nm which corresponds to a monolayer formation as the diameter of the hydrophobins are about 2 nm. For plain PS NPs, An increase in diameter is seen for HFBI adsorbing on the NPs whereas HFBII adsorption does not result in any increased particle size according to the DCS measurement. (Figure 2). PEI modified PS NPs are display a large spread of size indicating aggregation for both pristine NPs as covered NPs in MQ. (Figure 3)

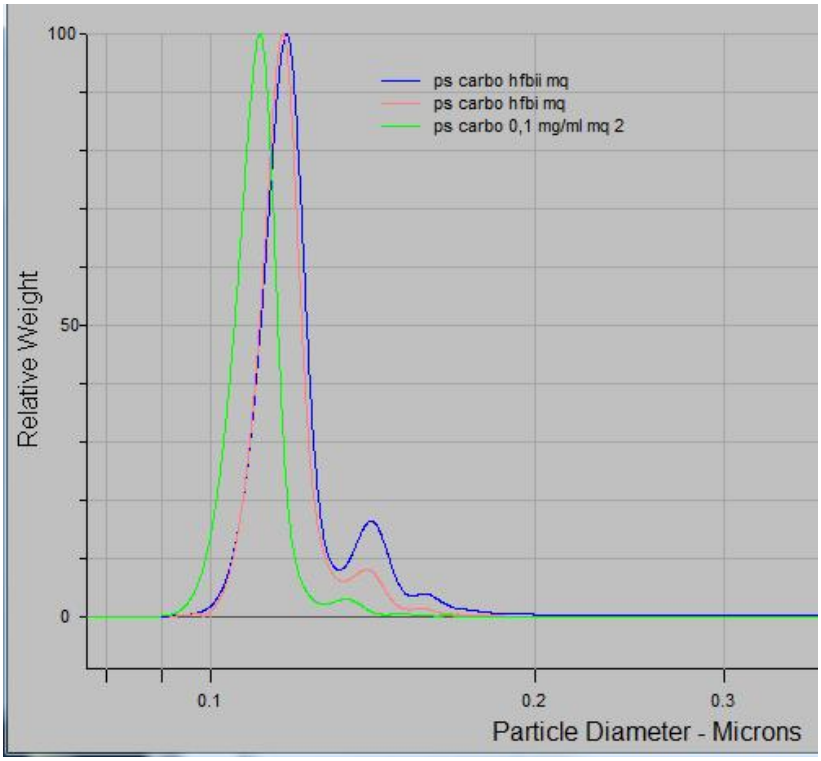


Figure 1 PS Carbo: HFBI, HFBII in MQ

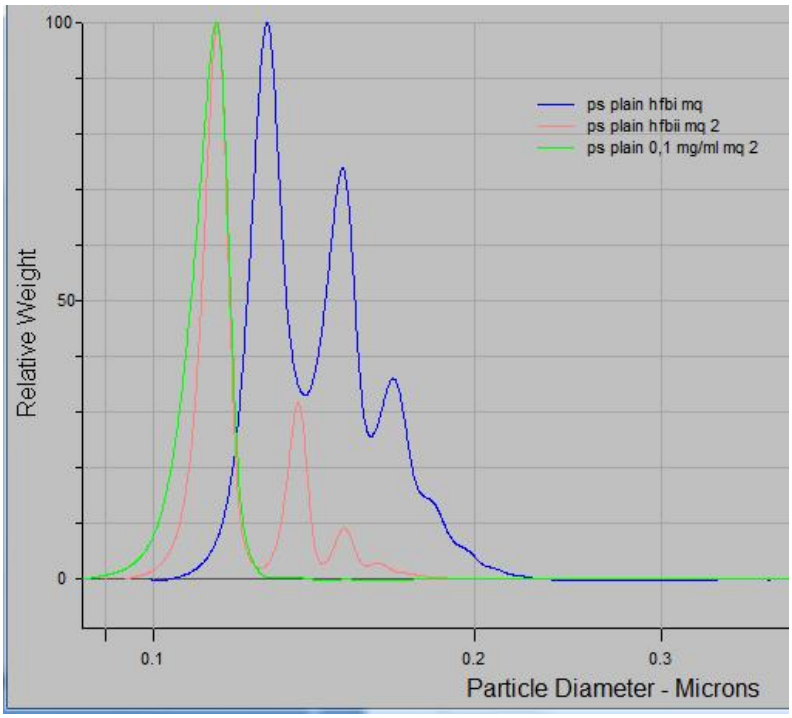


Figure 2 PS plain: HFBI, HFBII in MQ

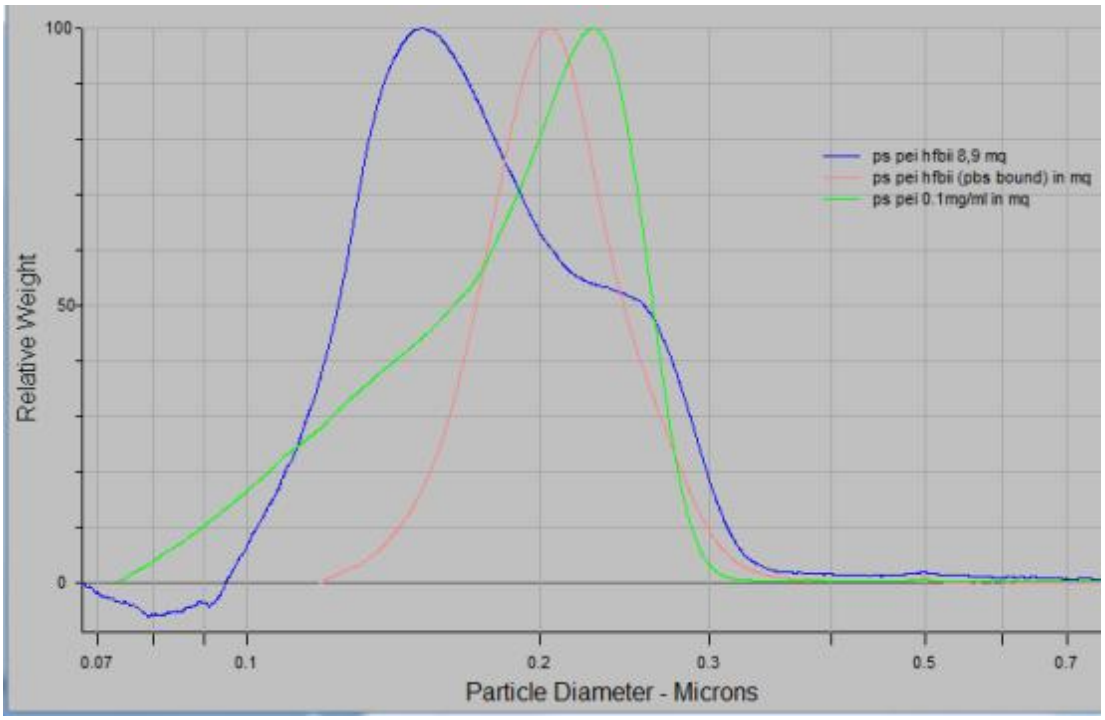


Figure 3 PS PEI, HFBI, HFBII in MQ

Effect of buffer

Comparison between dialysis samples run in PBS pH 7,4 or after final step of dialysis in MQ show a general decrease of aggregation to low levels when using MQ. Figure 4 shows the effect on PS plain and carboxylated PS NPs with HFBI and HFBII dissolved in PBS or MQ after dialysis. The results show a clear increase in particle size when using PBS as buffer. The spread of different sizes are also wider when using PBS compared to MQ. When comparing pristine NPs, (plain, carboxylated or PEI PS) in MQ and PBS, the results are similar, PBS results in increased aggregation and thus increased particle size (Figure 5)

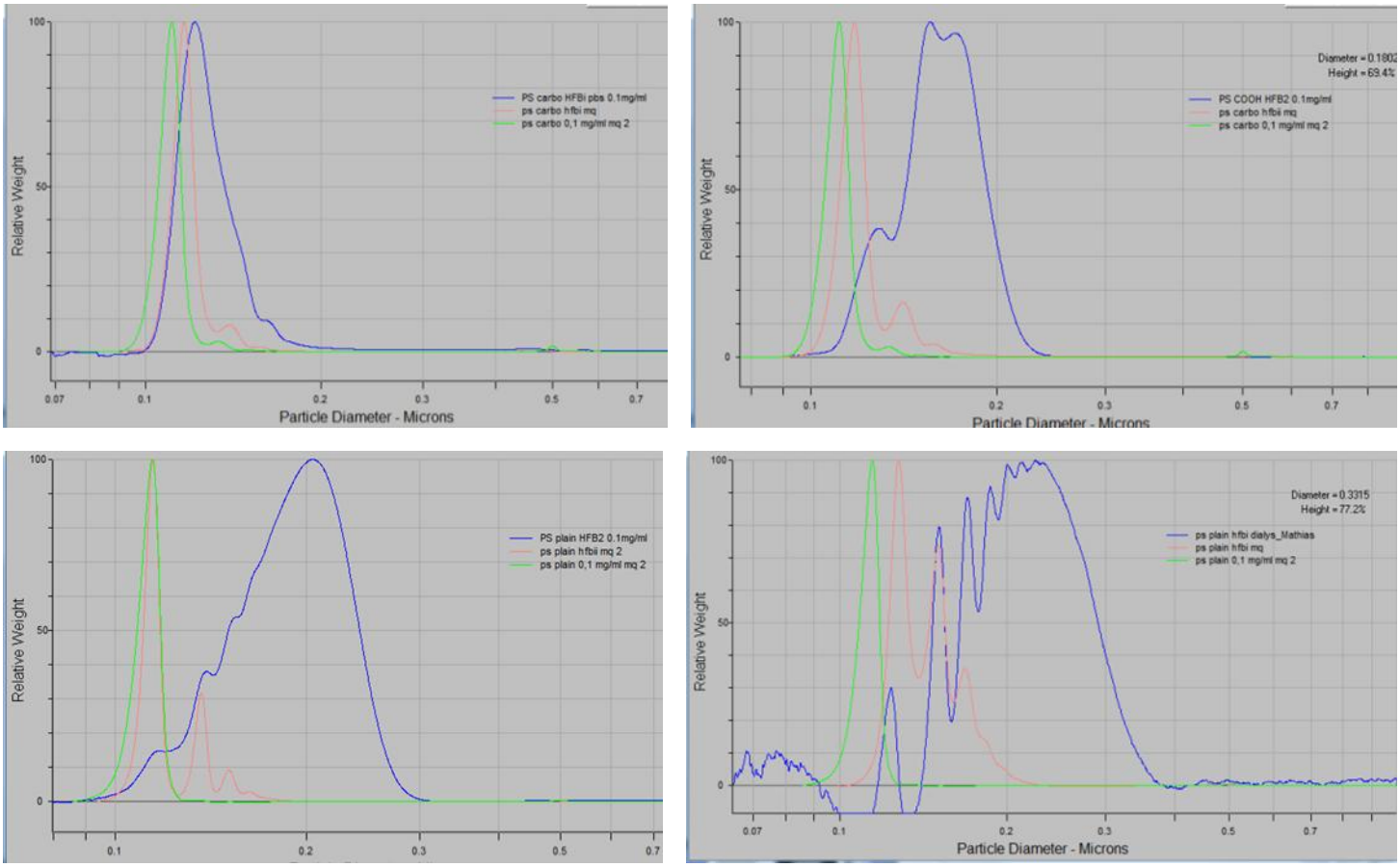
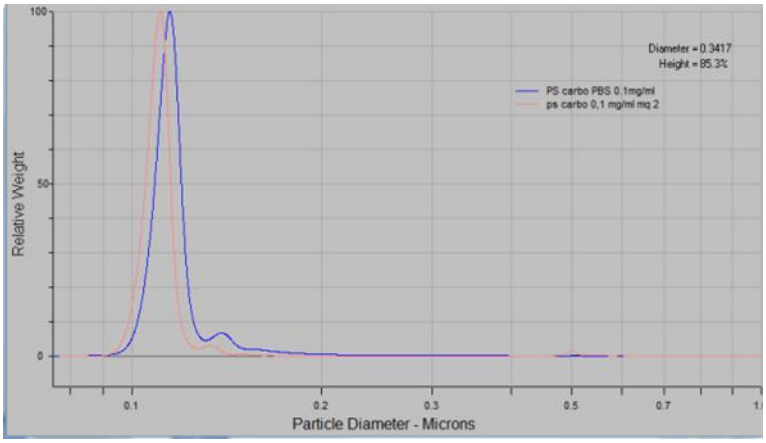
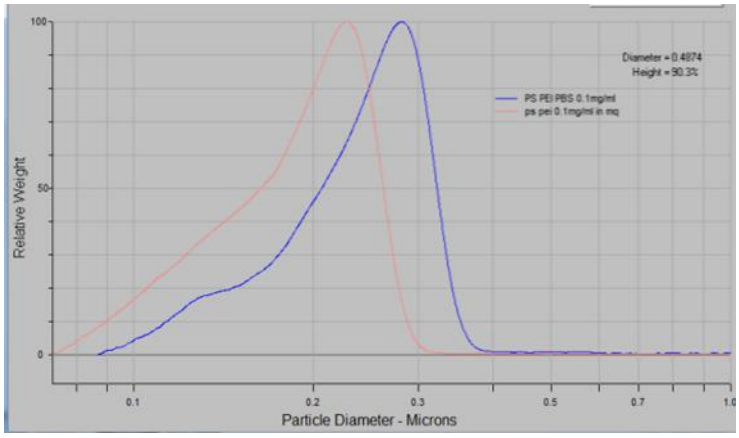


Figure 4. NPs with adsorbed HFB in MQ or PBS buffer





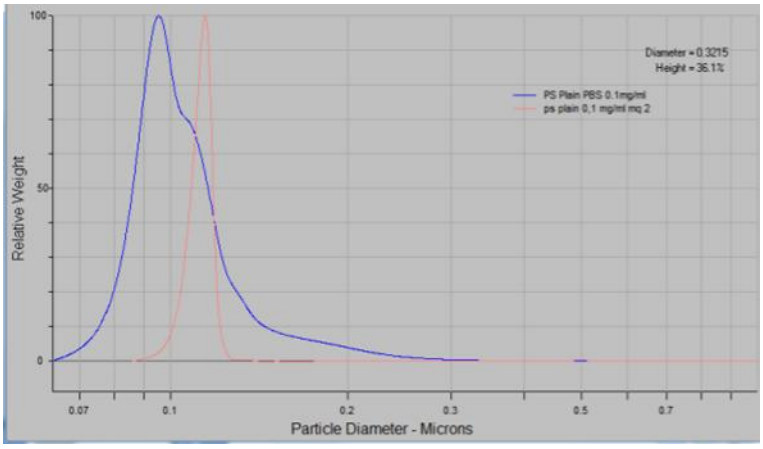


Figure 5. Pristine NPs in MQ or PBS buffer

DLS and Z-potential

DLS values for pristine NPs and NPs after hydrophobin adsorption in dialysis are shown in Table 1 and 2 for Carboxylated and Plain PS Nps respectively. The PDI value gives an estimate on the degree of aggregation of the particles and the number mean takes this into account.

The size of carboxylated PS NPs before and after hydrophobin adsorption can be seen in table 1. The elevated PDI value for HFBI-covered particles has been taken into account in the Number mean value. Here, an increase in diameter of 3,37 nm is measured. For HFBII, the PDI value is low and hence, the increase in diameter for both Z-average and Number mean diameter are similar, 5,13 and 4,29 nm respectively. As the diameter of HFBI and HFBII is around 2 nm (Linder, 2005), the adsorption of HFBI and HFBII on carboxylated PS NPs is about a monolayer thick.

On plain PS NPs, the PDI values are low and hence, very low levels of particle aggregation is interfering with the values of particle size (Table 2). The diameter is increasing after coating with HFBI with 12 to 8 nm which is closer to a double layer than a monolayer in particle size increase. For HFBII, the values are slightly higher, 13-10 nm in increased diameter after hydrophobin adsorption. Also here, a layer around a double layer is measured.

PS Carboxylated in MQ			
	Z-Ave (d.nm)	PdI	Number Mean (d.nm)
Pristine	101,90	0,02	83,57
HFBI	152,77	0,43	86,94
Increase in d	50,87		3,37
HFBII	107,03	0,03	87,86
Increase in d	5,13		4,29

Table 1. DLS size data for carboxylated PS NPs

PS Plain in MQ			
	Z-Ave (d.nm)	PdI	Number Mean (d.nm)
Pristine	96,61	0,00	79,67
HFBI	108,53	0,04	87,29
Increase in d	11,92		7,62
HFBII	109,87	0,02	89,75
Increase in d	13,25		10,09

Table 2. DLS size data for plain PS NPs

Preliminary experiments on Z-potential (Surface charge) of the particles were performed on NPs taken from Dialysis before final step in MQ. For HFBII there is a decrease in surface charge of covered particles compared to pristine in PBS for both plain and carboxylated PS Nps. The Z-potential of PS PEI NPs is largely unchanged before and after adsorption of HFBII (Table 3). The Z-potential of carboxylated PS NPs is lowered conciderably after adsorption of HFBI (Table 4). These results indicate that there is a protein layer of hydrophobin present on the NPs that is changing the surface charge of the particles.

NP	Pristine NPs Z pot (mV)	Covered NPs Z-pot (mV)	Change in Z-pot (mV)
PS plain	-64,775	-18,45	46,3
PS Carboxylated	-41,775	-9,775	32,0
PS PEI	20,125	22,125	-2,0

Table 3. Z-potential experiments in PBS pH 7,4, HFBII

NP	Pristine NPs Z pot (mV)	Covered NPs Z-pot (mV)	Change in Z-pot (mV)
PS Carboxylated	-41,775	-14,075	27,7

Table 4. Z-potential experiments in PBS pH 7,4, HFBI

SDS PAGE

1D SDS PAGE gel measurements showed here were performed after completed dialysis after the water step as well as after completed protein corona measurement. For protein corona, both pristine and post dialysis NPs were incubated with human plasma, washed and boiled before loading into gel. The Gel for

dialysis NPs can be seen in figure 6 and the gel for corona NPs can be seen in figure 7. The results of the gels are unfortunately of lower quality, with possible contamination bands associated with other proteins than hydrophobins visible. Nevertheless some comments can still be made from the gels. For HFBI, Bands can be seen from samples from both plain and carboxylated PS NPs. The band of the carboxylated NP is stronger. For HFBII , a strong band can be seen for hydrophobin adsorbed on plain and carboxylated NPS. A weak band of HFBII is also seen for the case where HFBII has been allowed to adsorb on the PEI modified PS NPs.

The gel for NPs after adsorption and following washing of human plasma protein corona is seen in figure 7. There are lower amounts and weaker bands of corona proteins on pristine Plain and PEI PS NPs compared to pristine Carboxylated NPs. This was also seen during the washing steps, the plasma proteins seemed more able to adsorb on the carboxylated Nps. The strongest case of protein corona proteins binding can be seen on post dialysis carboxylated NPs as a starting point. On the Plain NPs post dialysis the NPs with HFBI shows stronger bonds than the HFBII case where only low levels of proteins are present.

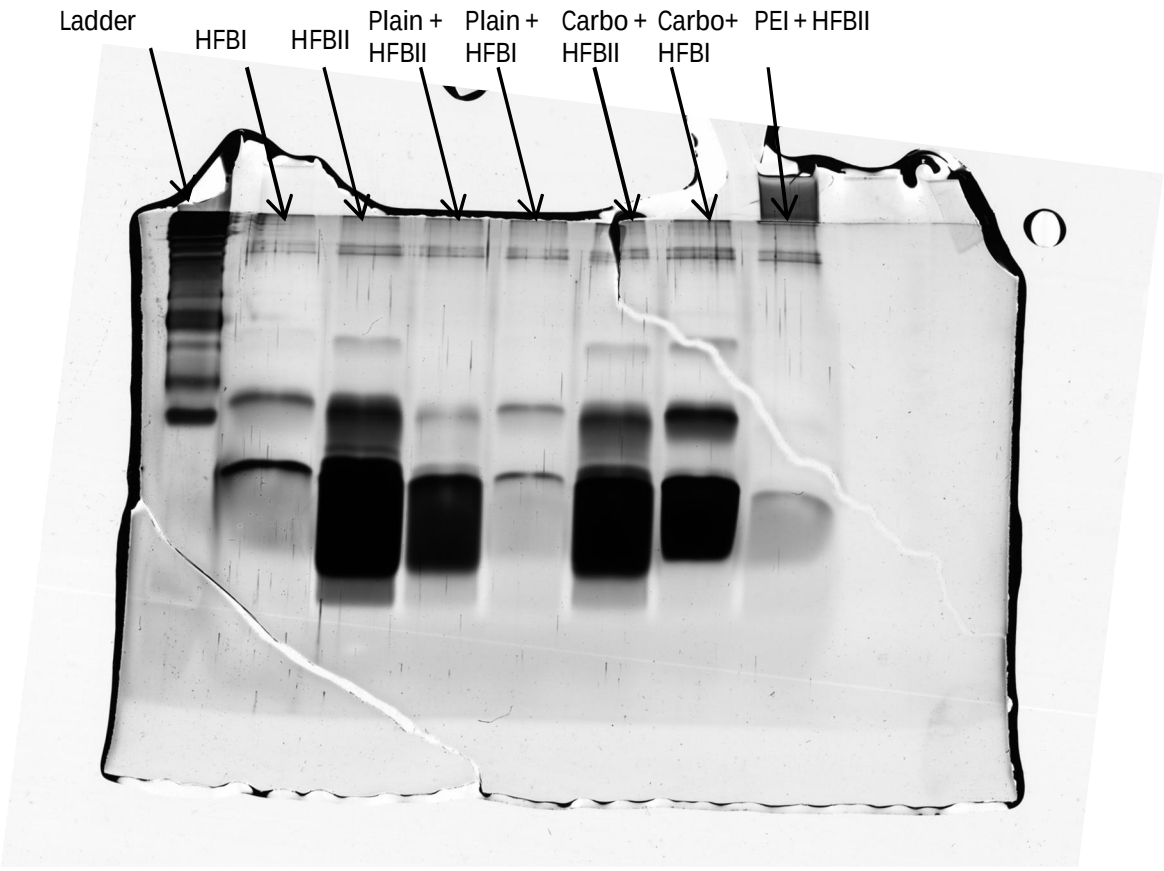


Figure 6. 1D SDS PAGE on post Dialysis samples

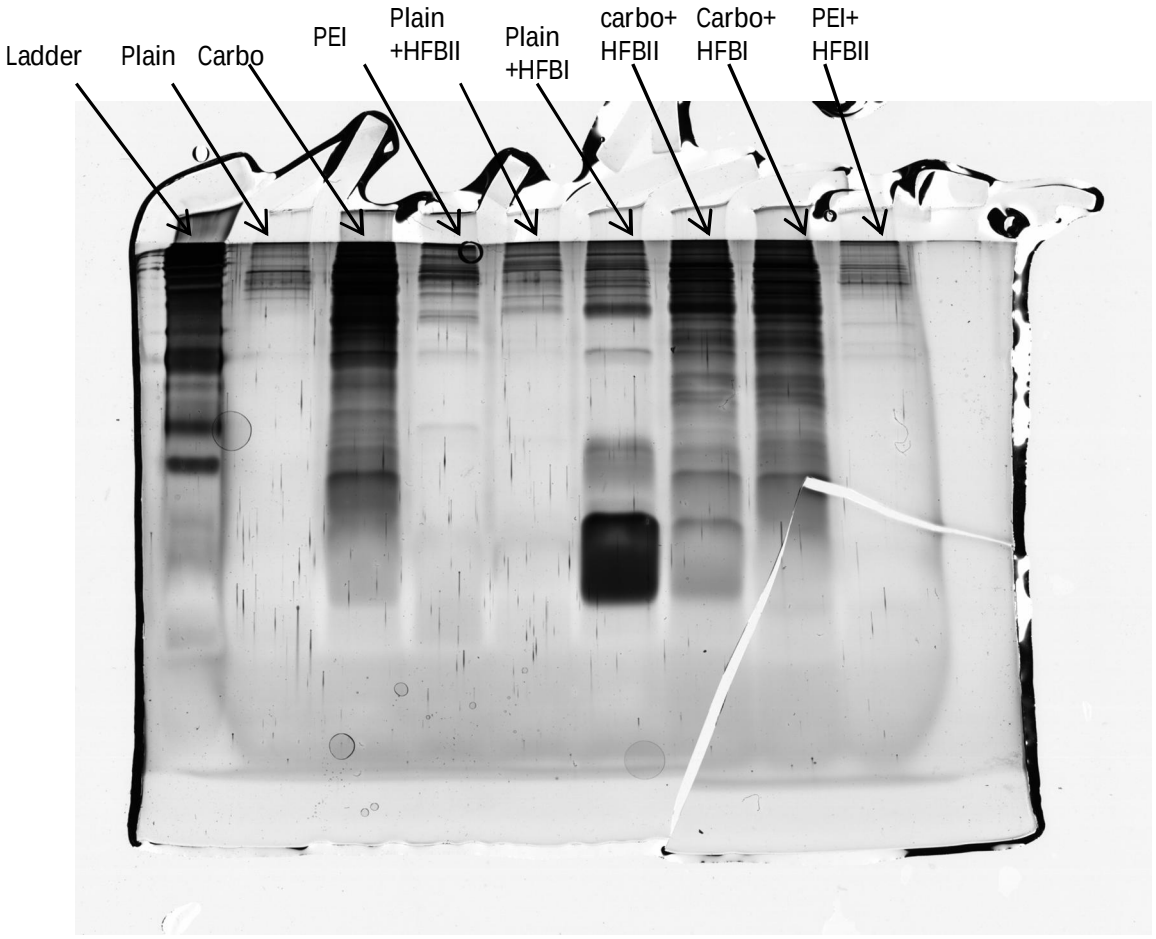


Figure 7 1D SDS-PAGE on Corona samples

4. Discussion and conclusions

HFBI was shown to increase the diameter of both plain and carboxylated PS NPs in both DCS and DLS experiments indicating a bound layer of hydrophobin on the particles about a monolayer to a double layer thick (below 10 nm). Preliminary experiments of the Z-potential of the particles in PBS were also altered considerably by the coverage of HFBI. In SDS-page, HFBI was present on the particles strengthening the claim that HFBI is adsorbing on the particles (gel output unfortunately of lower quality). For HFBII, the results were similar; DLS experiments indicated that HFBII is adsorbing on plain PS NPs where an increase in the diameter of the NPs of a about a monolayer is found for carboxylated PS NPs and a double layer for plain PS NPs. Also, the preliminary Z-potential measurements in PBS showed a conciderable change after hydrophobin adsorption on plain PS NPs strengthening the claim of an adsorbed hydrophobin layer. However the DCS experiments did not show any change in particle size after adsorption of HFBII on plain PS NPs, even as this has been found in previous experiments. Reruns are planned in this case. On carboxylated NPs with adsorbed HFBII, DCS, DLS and Z-pot indicated that HFBII is adsorbing on the particles, as found for HFBI. Also, SDS page indicated that HFBII is present on all NPs treated with the protein. On the positively charged PEI modified NPs, aggregation is causing problems in both covered and uncovered particles in preliminary results and further work is needed to solve this. Possibly nonionic surfactants, such as TWEEN is suggested to be used to avoid aggregation of covered particles, which are aimed to be hydrophobic after hydrophobin coverage.

Interestingly, all the covered and uncovered NPs were found to aggregate more or less when dissolved in PBS (DLS and DCS data). This was solved by changing the dialysis buffer to MQ water during the final day of dialysis. Continuing experiments are planned in order to finalize the study, including experiments on varying size.

The work described here will result in a future collaboration during autumn 2011 together with the same group and same author finalizing the work described here. As a result of this finalizing work, an article is planned to be produced.