Epitope Map Exchange Grant Report

Evaluation of the protein-corona structure of lead and bismuth piezoelectric nanoparticles

Margarita Fernanda Esquivel Gaón

fesquivel@cinvestav.mx

Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV)

University College Dublin (UCD)

Introduction

The functionalizing of nanoparticles (NP) provides high added value to nanomaterials used as bioprobes, nanosensors, drug-delivery agents, photocatalysts, piezoelectrics, etc. However, these functionalized properties may bring undesirable and increased reactivity with them when they enter in contact with biological systems. When the NP come in contact with different biological fluids (i.e plasma, surfactant) are covered by proteins and other biomolecules leading to a formation of the "protein corona". This interaction between NP and proteins is important not only for medical applications but also for evaluate the potential toxic effect of these particles. The type of proteins forming the protein corona depends on the NP physicochemical properties (size, shape, composition, charge) (Cedervall, Lynch et al. 2007; Lundqvist, Stigler et al. 2008; Monopoli, Walczyk et al. 2011; Sund, Alenius et al. 2011).

Has been proposed that the structure of the protein-corona can be divided into two parts: 1) the hard corona composed by proteins with major affinity for the surface and 2) the softer corona composed of those proteins with lower affinities and a rapid exchange dynamics between the biological fluids and the NP (Cedervall, Lynch et al. 2007). This last part of the protein-corona can lead to the exposure of new epitopes that alter the functionality of the protein, determining the particle interactions with biological surfaces and receptors; hence, the protein-corona gives a biological identity to the NP (Lynch, Salvati et al. 2009). To understand the protein-corona we must understand not only the composition and organization but also the kinetics affinities of association with the NP. This identity could influence the organ distribution and clearance as well as the cell uptake and distribution inside the cell (Lundqvist, Stigler et al. 2008; Nel, Madler et al. 2009).

The aim of this project is the determination of the protein-corona around two piezoelectric NP mainly formed by lead (PZT) and bismuth (BNT-BT), respectively. The mainly techniques used in

this study were Differential Centrifugal Sedimentation (DCS), Dynamic Light Scattering (DLS) and electrophoresis.

Nanoparticle Dispersion

In order to obtain a stable NP dispersion of the two piezoelectrics BNT-BT and PZT we used a protocol provided by UCD. This protocol consists in bath and tip sonication during 15 minutes followed by the addition of a stabilizing agent, in our case the protein albumin, to continue with the protocol we centrifuged two times at different speeds (27 and 200 x g) and we kept the supernant that contain the particles that are ≤ 200 nm.

Size distribution of nanoparticles in biological media

The method of choice to assess size distribution of NPs in this case BNT-BT and PZT dispersed in biological fluids (*in situ*) is the DCS. With this technology we can analyze in high detail changes in the size distribution of NPs over time or in different media. We used this approach, together with DLS to assess the stability of the dispersions generated with the dispersion protocol established in UCD for BNT-BT NPs. Firstly we measured whether the sucrose gradient used by the disc centrifuge to resolve the size of NPs, prepared dissolving the sucrose in either water, phosphate buffer (PB) or phosphate buffer saline (PBS) affects the separation of the BNT-BT and PZT by the CPS disc centrifuge; PB is the dispersant used to generate the dispersions, PBS though has a salt composition comparable to the cell culture medium used in the *in vitro* exposure experiments, therefore it would be the appropriate solvent for the sucrose gradient when assessing the size distribution of NPs in the same conditions of *in vitro* exposure (fig 1).

A)

B)

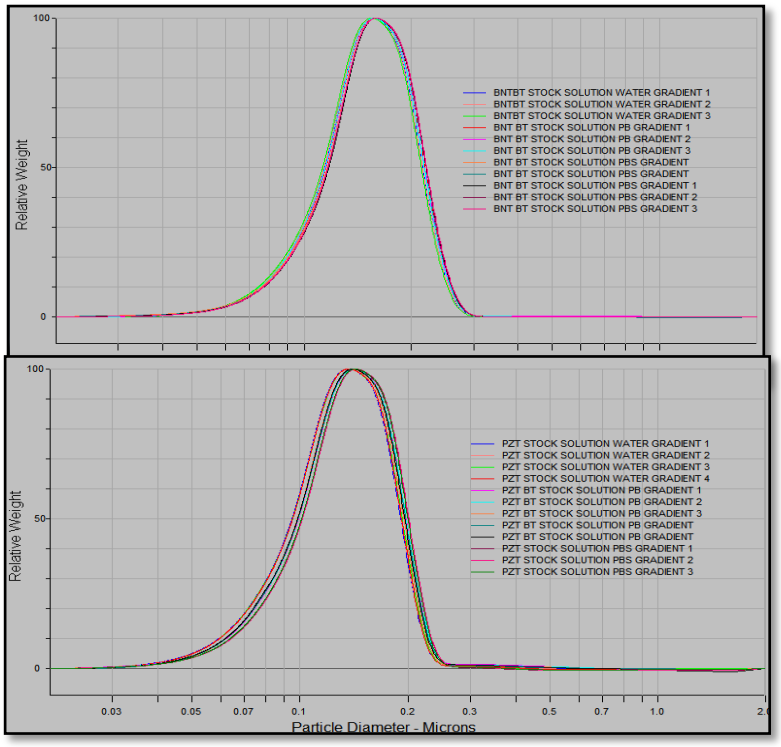


Fig.1. Gradient composition does not affect size distribution of A) BNT-BT and B) PZT NPs. The size distribution of BNT-BT and PZT NPs dispersed was analyzed by DCS using a 24%-8% sucrose gradient prepared in H_2O, PB or PBS.

This control experiment performed on both NPs

showed that the size distribution is comparable in the three gradients; therefore we can use one gradient, with sucrose dissolved in PBS, to perform all experiments (fig 1A and B).

We couldn't observe any alterations in the size distribution of the NPs in the different gradients; therefore, we can conclude that the gradient composition does not affect the measurement using DCS.

Our next step was to study the stability of the BNT-BT and PZT dispersions over time to assess whether the aggregation state changes with aging of the dispersion. We used DCS and DLS to measure the size distribution and average size over 15 days; NP dispersions were vortexed before the measurement. According to the data obtained with DLS (fig. 2) indicate that both of the NPs maintain a consistent average size over 15 days and therefore the same suspension can be used for this period of time. The tables 1 and 2 show the average size obtained by DCS and DLS techniques.

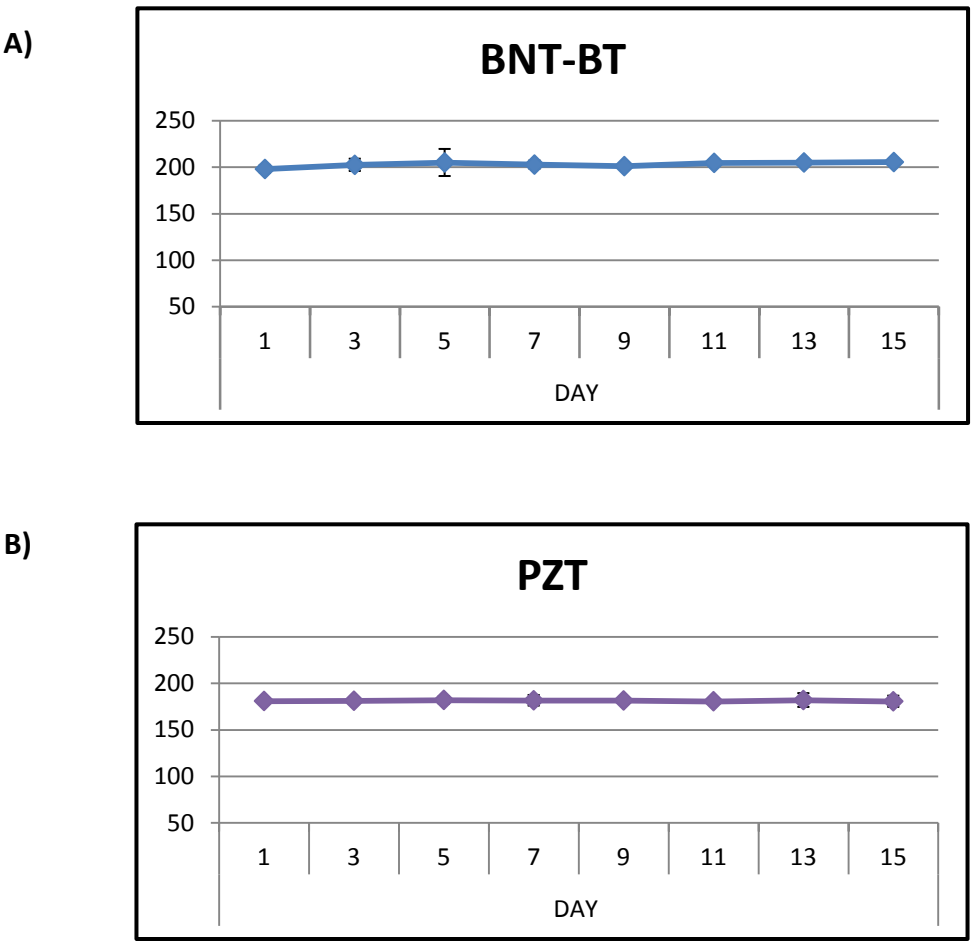
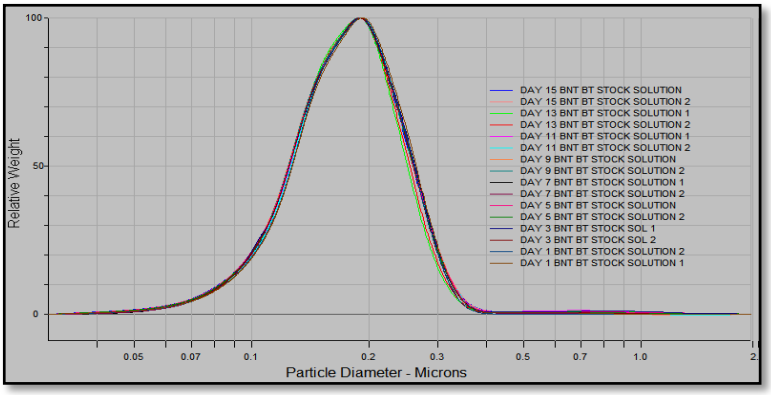


Fig.2. NPs dispersions show reproducible size distribution over 15 days by DLS. Average size of A) BNT-BT and B) PZT NPs dispersed according to the protocol provided by UCD was analyzed by DLS over 15 days. The graphs show the mean average size ± standard deviation, n=6.

In order to corroborate this data, we performed the analysis of the NPs dispersion with the use of DCS (fig 3 and 4). The results obtained show that the size distribution of BNT-BT and PZT dispersions is stable during the period of study (15 days).

A)



B)

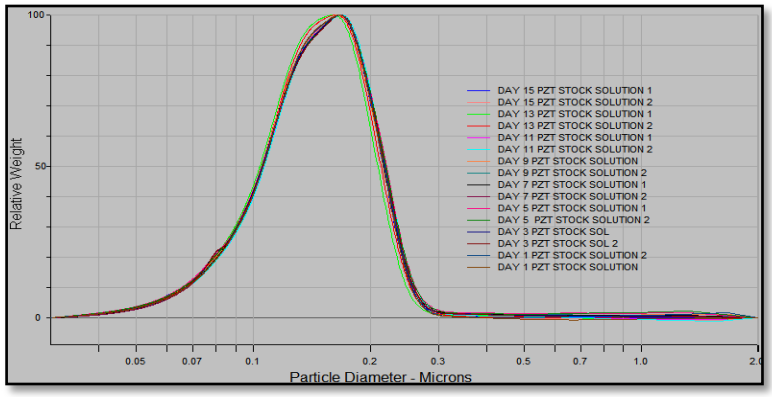


Fig.3. NPs dispersions show reproducible size distribution over 15 days by DCS. Size distribution of A) BNT-BT and B) PZT NPs dispersed according to the protocol provided by UCD was analyzed by DCS over 15 days

A) **B)**

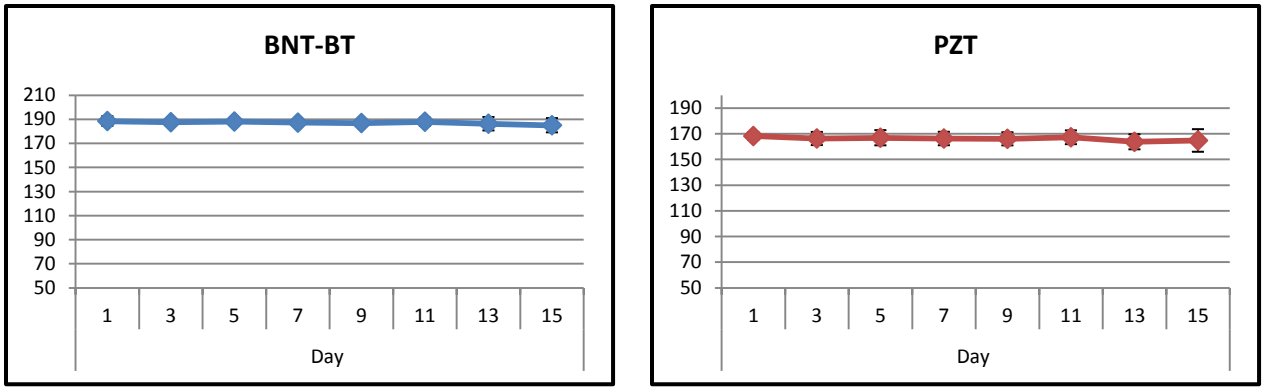


Fig.4. NPs dispersions show reproducible size distribution over 15 days by DCS. The graphs indicate the mean ± standard deviation of the peak in the size distribution obtained by DCS for A) BNT-BT and B) PZT NPs dispersion, n=6.

The measurements show that the size distribution of NPs doesn't change significantly, therefore the dispersions can be considered stable for a period of 15 days.

Table 1. Average size values of BNT-BT NPs dispersion, measured by DLS and DCS. The expressed values in the table are mean ± standard deviation, n=6.

Day	Average-size DCS	Average-size DLS
1	188.48 ± 4.03	197.98 ± 4.31
3	187.55 ± 2.56	202.41 ± 6.57
5	188.1 ± 2.63	204.94 ± 14.54
7	187.16 ± 2.54	202.70 ± 4.33
9	186.85 ± 1.67	201.04 ± 1.43
11	187.95 ± 3.37	204.56 ± 2.78
13	186.28 ± 5.58	204.98 ± 0.06
15	185.01 ± 5.91	205.43 ± 2.16

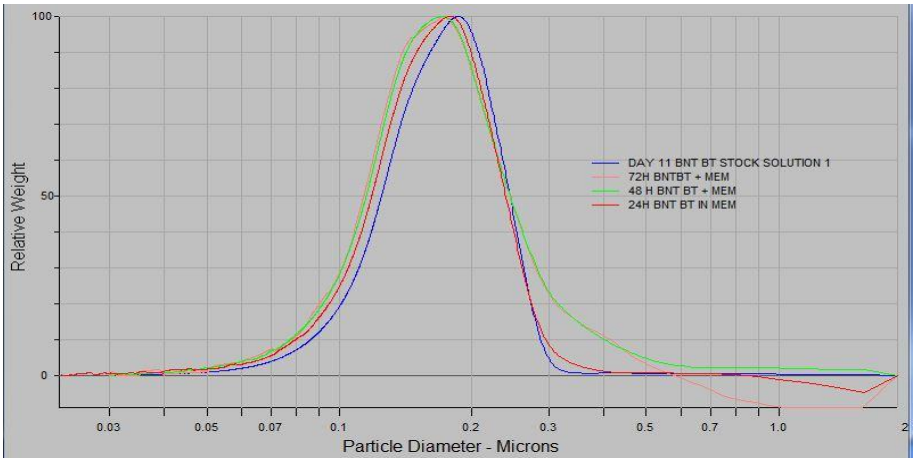
Table 2. Average size values of PZT NPs dispersion, measured by DLS and DCS. The values expressed are the mean ± standard deviation, n=6.

Day	Average-size DCS	Average-size DLS
1	168.16 ± 2.92	180.8 ± 3.25
3	166.25 ± 5.11	181.03 ± 2.96
5	166.85 ± 5.91	181.94 ± 3.93
7	166.23 ± 5.23	181.53 ± 5.70
9	166 ± 5.09	181.35 ± 2.14
11	167.2 ± 5.37	180.51 ± 0.40
13	163.66 ± 5.84	181.81 ± 7.42
15	164.71 ± 8.79	180.48 ± 6.01

To correlate the size distribution to the available dose administered to cells in culture we have performed DCS measurements of BNT-BT and PZT NPs dispersed in complete cell culture medium (MEM) supplemented with 10% fetal bovine serum (FBS) at the highest concentration administered to cells, 100 µg/ml the analysis was performed at 24, 48 and 72 hours, NP dispersions were vortexed before the measurement.

Incubation of BNT-BT and PZT NPs in cell culture medium supplemented with FBS generated a small variation of the size distribution. The dispersions show smaller sizes within a 20 nm range; what needs to be noted is that the size distribution profiles recorded by the DCS become less sharp with longer incubations, which is consistent with sedimentation of the NPs in the tube (Fig. 5).

A)



B)

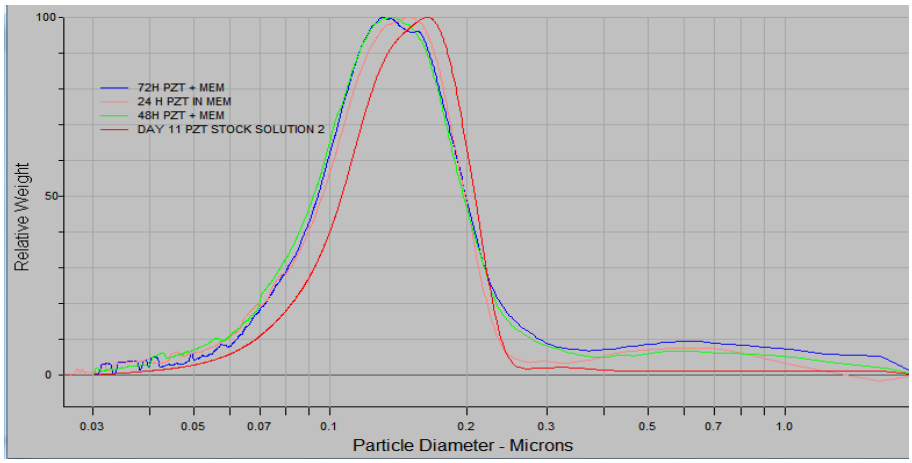


Fig.5. BNT-BT and PZT NPs maintain a similar size distribution in conditions that mimic exposure to *in vitro* cell cultures.

Size distribution of BNT-BT and PZT NPs dispersed according to the protocol provided by UCD was analyzed by DCS after incubation at a concentration of 100 µg/ml in cell culture medium supplemented with 10% FBS. The measurements show a small reduction in the size distribution of NPs; the recorded size distribution becomes less stable with time due to sedimentation of the NPs in the tube.

Protein Corona

The protein corona is formed when the NP come in contact with fluids that contain proteins, lipids and other biomolecules, this could change the way that they interact with the environment, for that reason our experiments are were design to have a better understanding of the proteins that form the protein corona in both piezoelectric BNT-BT and PZT NPs.

In order to obtain a higher concentration of proteins in these studies we centrifuge the 1 mg of the NP dispersion and eliminate the supernatant, and then we incubate with different concentrations (10, 55 and 100%) of FBS and plasma (6, 33 and 100%) during 1 hour. In the next steps we try to recover the particles and the hard protein corona that is around them, removing the unbounded proteins, for that we use centrifugation steps at 14,000 rcf during 45 minutes and 4 washes with PBS. The hard corona was removed from the particles using denaturing conditions and separated by 1D PAGE. We use plasma and FBS under the same treatment as the experiment, as a control of background.

The figure 6 shows the gel where the BNT-BT NP were incubate with 0, 6, 33 and 100 % of plasma or 0, 55 and 100 % of FBS during 1 hour. If we incubate the NP with plasma, the BSA around the NP from the dispersion, is replace by another protein. Instead, if we incubate the NP dispersion with FBS we can see that the amount of BSA it is not affected, however we can notice an increase in the amount of proteins that form part of the hard protein corona.

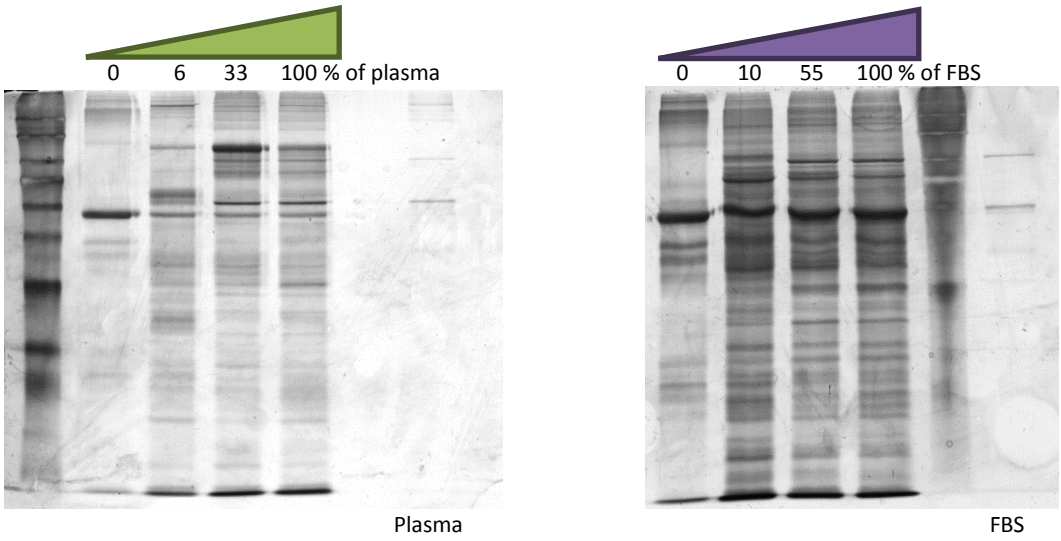


Figure 6. Profile of proteins attached to the BNT-BT NP after incubation with a) Plasma and b) FBS during 1 hour.

In the figure 7 we can see the hard corona of the PZT NPs after 1 hour incubation with 0, 6, 33 and 100 % of plasma and 0, 55 and 100 % FBS during 1 hour. The profile shows an increase in the number of proteins as the concentration of the plasma or FBS increase.

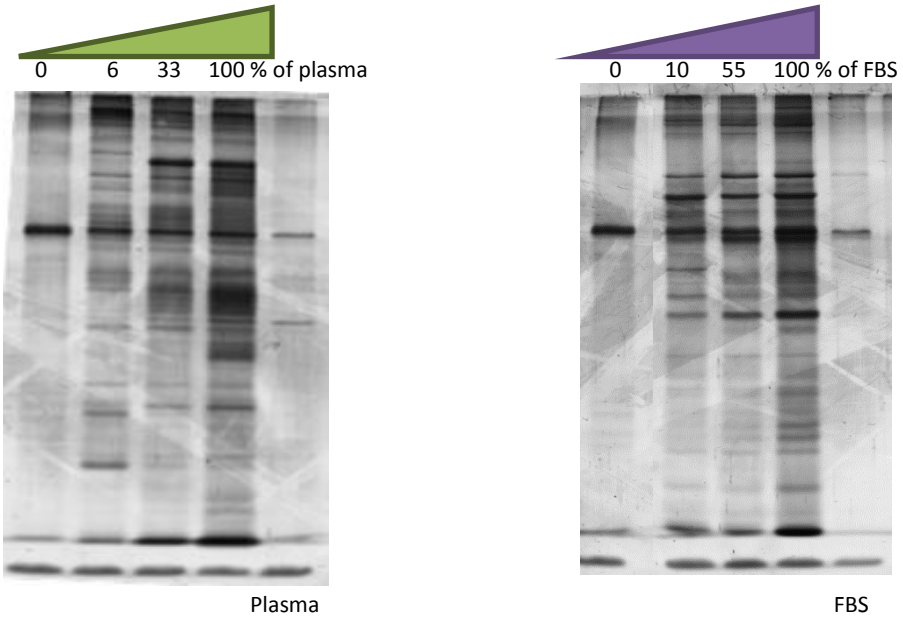


Figure 7. Profile of proteins attached to the BNT-BT NP after incubation with a) Plasma and b) FBS during 1 hour.

Conclusion

- If we use the dispersion protocol provided by UCD we can obtain dispersions of BNT-BT and PZT NP in the range ≤ 200 nm.
- The average size for BNT-BT particles in the dispersion by DCS is 188.48 nm while the average size using DLS is 197.98 nm.
- Using the DCS the average size for PZT particles in the dispersion is 168.16 nm but if we use the DLS the average size is 180.8 nm.
- Using the dispersion protocol from UCD we can obtain a BNT-BT and PZT dispersion stable for 15 days.
- The hard protein corona around BNT-BT and PZT NPs is formed mainly by the BSA used in the dispersion protocol.
- The BSA around the BNT-BT NPs can be exchange with other proteins with mayor affinity when we incubate during 1 hour with plasma.
- The protein corona formed when we add the BSA into the PZT NP dispersion is not removed by the proteins in the plasma or FBS; however, other proteins can interact with the particles and form part of the hard corona.

Future Work

- Instead of using albumin as a dispersant we will try to use plasma and FBS in a final concentration of 10 mg/ml.
- Identify the proteins that are part of the protein corona either with plasma or FBS using Mass Spectrometry.

References

Cedervall, T., I. Lynch, et al. (2007). "Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles." Proceedings of the National Academy of Sciences of the United States of America **104**(7): 2050-2055.

Lundqvist, M., J. Stigler, et al. (2008). "Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts." Proceedings of the National Academy of Sciences of the United States of America **105**(38): 14265-14270.

Lynch, I., A. Salvati, et al. (2009). "Protein-nanoparticle interactions: What does the cell see?" Nature nanotechnology **4**(9): 546-547.

Monopoli, M. P., D. Walczyk, et al. (2011). "Physical-chemical aspects of protein corona: relevance to in vitro and in vivo biological impacts of nanoparticles." Journal of the American Chemical Society **133**(8): 2525-2534.

Nel, A. E., L. Madler, et al. (2009). "Understanding biophysicochemical interactions at the nano-bio interface." Nature materials **8**(7): 543-557.

Sund, J., H. Alenius, et al. (2011). "Proteomic characterization of engineered nanomaterial-protein interactions in relation to surface reactivity." ACS Nano **5**(6): 4300-4309.