Polystyrene nanoparticles interactions with Calbindin D_{9k} and Monellin

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Abstract
Nanomedicine and the use of nanoparticles (NPs) are growing like never before. It is therefore important to investigate the interactions in the nano world and the possible hazards. Proteins in organisms can adsorb to NPs and change the proteins structure and therefore their functions. This could be a potential danger and thus an interesting research field. Here, we explore how the proteins Calbindin D_{9k} and Monellin interacts with polystyrene NPs using fluorescence spectroscopy and NPs colloidal stability using dynamic light scattering (DLS). We show that similar charges repel and the opposite attract proteins to NPs, proteins hydrophobic patches allow them to adsorb to hydrophobic NPs and the size of the NPs seems to affect how fast the proteins adsorbs to the NPs.

1. Introduction

Some people say that what we cannot see does not exists. Today, social media are letting us see what is going on all around the world, things that 10 years ago we could not see but still existed. However, there are things we cannot see but does exists, not 5 000 km away but right where we live. We are exposed to these things every day, whether we are brushing our teeth (Galletti et al., 2016), sun bathing (Sendra et al., 2016) or walking down the street (Lee et al., 2012). These things are called nanoparticles (NPs) and by definition they have at least one dimension ranging from 1-100 nm or their volume-specific surface area is greater than 60 m^2/cm^3 (Rauscher et al., 2013). The NPs in products we use ever
day can make their way through the sewage systems and end up in lakes and oceans (Westerhoff et al., 2011) and harm the marine life (Ziccardi et al., 2008). NPs up rise in usage is leading to greater exposure e.g. by inhalation or ingestion (Kumar and Dhawan, 2013). Exposure of NPs has been shown to hinder cell cycle (Kansara et al., 2013), induce inflammatory responses (Giovanni et al., 2015) and oxidative stress (Manke et al., 2013). However, NPs can also be used to improve our health. The field of nanomedicine has been growing the past decade (Emerich and Thanos, 2006), especially because NPs can help with targeting specific cells and medicines solubility (Riehemann et al., 2009). Therefore, NPs have successfully been used to help treat cancer in vitro (Hu et al., 2010). In vivo though, the NPs will encounter a variety of organic molecules, especially proteins, that interacts with the NPs in unknown ways. How they interact depends on the NPs size, surface charge and shape (Walkey and Chan, 2012). Proteins differs just like NPs in size, surface charge and shapes, this makes predicting their interactions problematic. Their interactions could alter the proteins conformation (Cukalevski et al., 2011) and therefore their function (Karlsson and Carlsson, 2005). The purpose of this study is to lay

out groundwork for how proteins and NPs interact. Mutants with different charge and hydrophobicity of Calbindin D_{9k} and Monellin and how they interact with carboxyl group surfaced polystyrene NPs is investigated. Calbindin D_{9k}, a Ca^{2+}-binding protein of the calmodulin superfamily and Monellin, a sweet-tasting plant protein were chosen for this study because charge modifications do not alter their shape or stability (Assarsson et al., 2014; Fast et al., 2001; Xue et al., 2006).

2. Materials and methods

2.1 Materials:

Our procedure followed Nasir et al. (2015)

2.1.1 Buffer

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was dissolved in filtered water to reach the final concentration of 10 mM and the pH was set to 7.4 to imitate the physiological pH. The proteins do not denature and the NPs do not aggregate in HEPES buffer and was therefore used throughout the experiment.

2.1.2 Fluorophores

Nile Red (NR) was dissolved in dimethyl sulfoxide (DMSO) to get the final concentration of 31,8 μg/ml. NR is a hydrophobic molecule (see figure 1).

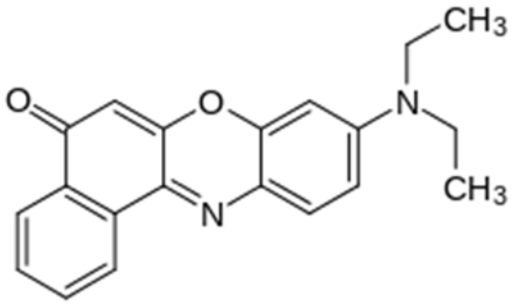

Figure 1: Chemical structure of Nile Red. https://en.wikipedia.org/wiki/Nile_red#/media/File:Nilrot.svg (2016-12-22)

8-Anilinonaphthalene-1-sulfonic acid (ANS) was dissolved in filtered water with the final concentration of 1, 3 mg/ml. ANS is a hydrophobic molecule with partial negative oxygens (see figure 2).

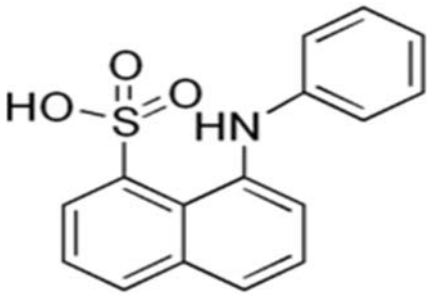

Figure 2: Chemical structure of ANS. https://en.wikipedia.org/wiki/8-Anilinonaphthalene-1-sulfonic_acid#/media/File:Phenylperi_acid.png (2016-12-22)

The fluorophores concentrations were calculated to theoretically cover all the NPs surface area.

2.1.3 Proteins

The wildtype and two mutants of Calbindin D_{9k} (Bovine) were used: the wildtype M0 and two mutants M11 (E26Q, E17Q and D19N) and M56 (F10A and P34M). The total charge of M0, M11 and M56 is -7, -4 and -7 respectively and M56 is less stable than M0. 3 mutants of Monellin were used: -2 (C41S, Q13E, N14D, Q28E and N50E), +2 (C41S and M42L) and +8 (C41S, E2Q, E4Q, D12N, E22Q, E48Q and E59Q). Monellins mutants name corresponds to their total charge. The proteins were dissolved in HEPES buffer and centrifuged. The absorbance of the proteins was measured at 280 nm with a spectrophotometer, because the three aromatic amino acids (phenylalanine, tryptophan and tyrosine) have their absorbance maximum at this wavelength. The concentration was determined by the formula $C = ABS/\varepsilon \times l$ where C = concentration, ε = extinction coefficient, l = length of the cuvette and ABS = absorbance. The extinction coefficient for Calbindin D_{9k} is 1490 $M^{-1}.cm^{-1}$ and for Monellin 14600 $M^{-1}.cm^{-1}$ (Nasir et al., 2015). The proteins were expressed in *Escherichia coli*.

2.1.4 Particles

Three different sized carboxylated polystyrene nanoparticles (P1 ~ 24 nm, P2 ~ 47 nm, P3 ~ 100 nm) used in this paper were purchased from Bang Laboratories. Inc. and Polyscience. They were already dialyzed against filtered water to remove surfactants, that is used to keep the NPs from aggregating, filtered and the concentration determined when we started working with them. The concentrations of NPs stock solutions were 6,5 mg/ml, 15,5 mg/ml and 59 mg/ml for P1, P2 and P3 respectively.

2.1.5 Plates

Polystyrene 96 half-area well plates with clear bottom and non-binding surface (Corning 3881) was used throughout the screening experiments.

2.2 Methods (general)

2.2.1 Dialyze

This method is used to reduce e.g. surfactants, ion or alter the pH in a solution and it is based on osmosis. By enclosing the solution inside dialyze tubes and placing the tubes in e.g. filtered water or buffer the molecules will diffuse along its concentration gradient. The dialyze tubes are made of a semi-permeable membrane and tubes with varied sizes of pores can be used to separate different sizes of molecules.

2.2.2 Spectrophotometer

A specific wavelength of light is focused through a cuvette containing a sample to measure the absorbance. Depending on what molecules absorbance is being measured the wavelength can be altered. This is because molecules absorb different wavelengths.

2.2.3 Dynamic light scattering (DLS)

DLS directs light through a sample and due to the particles, the light is scattered and detected. The scattered light can either interfere constructively or destructively. By detecting light scattering intensity over time, data can be obtained about the particles hydrodynamic diameter.

2.2.4 Fluorescence spectroscopy

Light is focused onto samples to excite e.g. fluorophores and the emission intensity is detected. The excitation light and emission detection can be set to different wavelengths depending on the fluorophore.

2.3 Method (specific)

2.3.1 Full screening analyses

Fluorophores were used to detect any interactions between the NPs and the proteins. The fluorescence intensity will increase and the emission wavelengths will shift when the fluorophores ANS and NR moves from a hydrophilic to a hydrophobic environment. The polystyrene NP has negative carboxyl groups (COO^-) bound to the hydrophobic polystyrene. Samples with fluorophore only, protein + fluorophore and particle + fluorophore was used as controls for the samples containing protein + fluorophore + particle. Indications of the interactions can be found by comparing the fluorophore intensity of these samples. There are six possible scenarios. (a = the fluorophore does NOT adsorb to the particle, b = the fluorophore does adsorb to the particle). (See figure 3).

Scenario 1a:

The proteins adsorb to the particle and thus the fluorophores are released from the protein. The fluorescence intensity (IF) is lower for the protein + fluorophore + particle (total) than the IF from the protein + fluorophore (protein) combined with the IF from the particle + fluorophore (particle).

Scenario 2a:

The proteins adsorb to the particle, the proteins conformational shape alters and the fluorophores can therefore bind to the proteins. IF_{total} is greater than $IF_{protein} + IF_{particle}$.

Scenario 3a:

The proteins do not adsorb to the particle and the fluorophores do not bind to the protein. IF_{total} is equal to $IF_{protein} + IF_{particle}$.

Scenario 1b:

The proteins adsorb to the particle and with greater affinity than the fluorophores. IF_{total} is less than $IF_{protein} + IF_{particle}$.

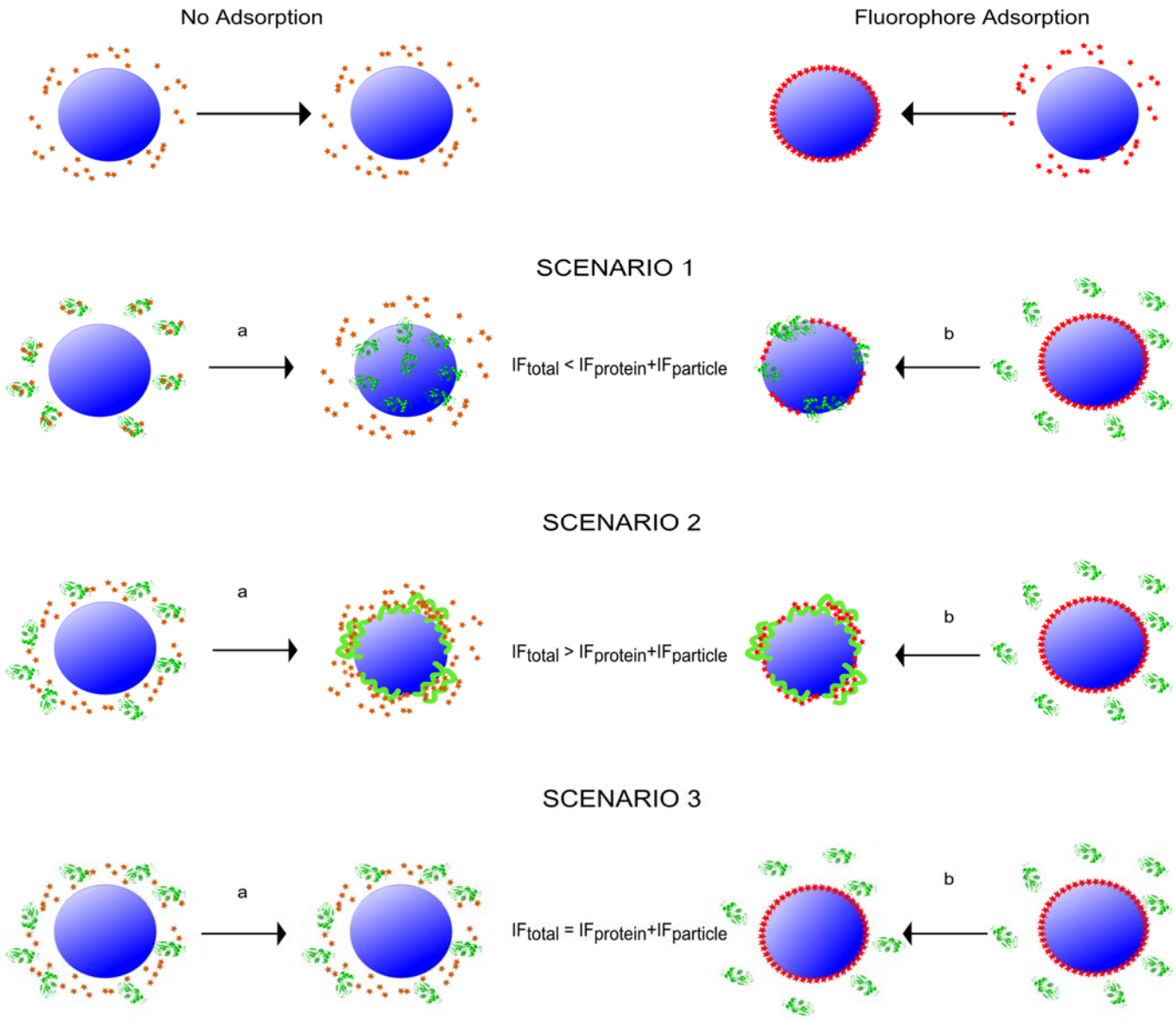

Figure 3: 6 different scenarios of how fluorophores, NPs and proteins could interact and how the fluorescence intensity from the samples protein + fluorophore, particle + fluorophore and protein + fluorophore + particle can be interpreted into these 6 scenarios.

Scenario 2b:

The proteins and fluorophores adsorbs to the particle, the proteins conformational shape alters and the fluorophores can therefore additionally bind to the protein. IF_{total} is greater than $IF_{protein} + IF_{particle}$.

Scenario 3b:

The proteins have less affinity to adsorb to the particle than the fluorophores. IF_{total} is equal to $IF_{protein} + IF_{particle}$.

2.3.2 Fluorescence measurements

The fluorescence intensity was measured for each sample, 3 replicates per sample from the same stock solution, using a fluorescence plate reader (BMG FLUOstar) directly after the stock solutions were mixed. Stock solutions were made by first adding HEPES buffer, fluorophore, NP and last protein. The concentrations of the NPs were calculated to get equivalent surface area of the three different sized NPs. For the NR experiments the excitation wavelength was set to 550 nm and emission wavelengths to 590, 630 and 660 nm to detect NRs fluorescence wavelength shift when bound to hydrophobic patches. For the ANS experiments the excitation wavelength was set to 320 nm and emission wavelengths to 460 and 475 nm to detect ANS fluorescence wavelength shift when bound to hydrophobic patches. Temperature was set to 37°C to imitate the physiological temperature and because the fluorescence plate reader cannot keep a steady temperature under ~ 30°C.

2.3.3 DLS measurements

The hydrodynamic diameter of the NPs in each well, 3 replicates per sample, was measured using a DLS plate reader (DynaPro Platereader-II, Wyatt Technology, CA, USA). The measurements were made with a 158° scattering angle at 25°C, acquisition time was set to 5 seconds with 10 acquisitions per well. DLS measurements were done approximately 16 – 20 hours after the stock solutions were mixed because fluorescence measurements were done prior to the DLS measurements.

3. Results

3.1 Full screening with NR

To detect interactions between the 3 NPs and the 6 proteins with NR a plate plan was designed and fluorescence intensity was measured. (See plate plan 1 in appendix for specifics). NR adsorbs to all particle sizes and therefore is only scenarios (b) considered.

3.1.1 P1 (See Figure 4)

M0 does not adsorb to the particle. M11 and M56 do adsorb to the particle. -2, +2 and +8 have greater affinity for the particles than for NR.

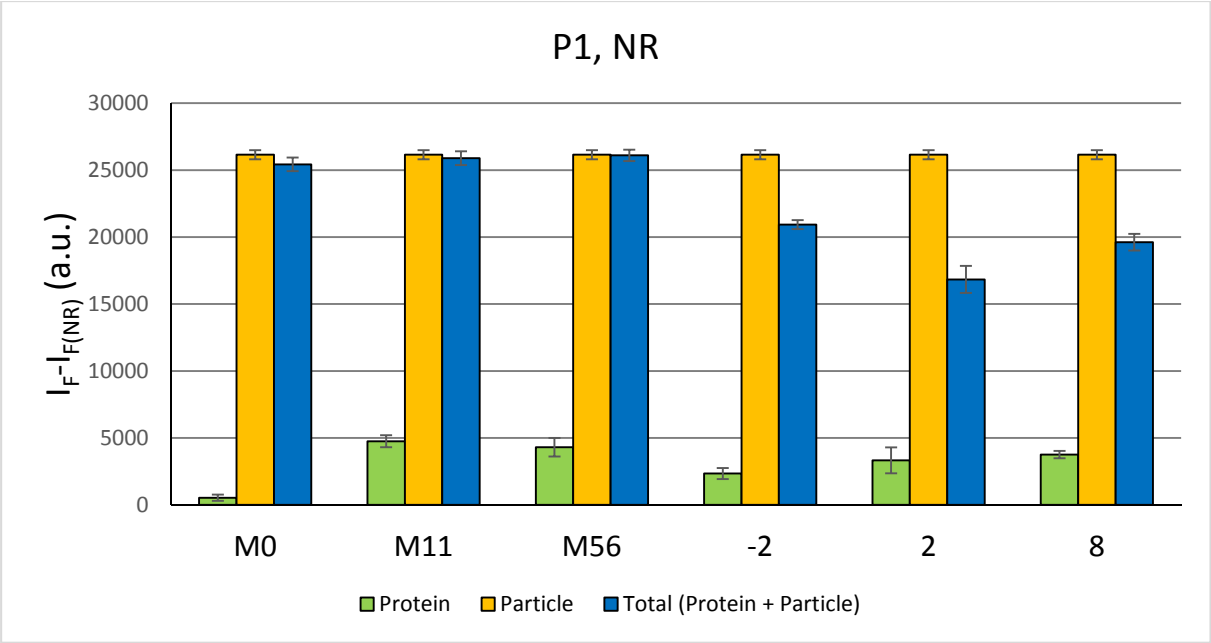

Figure 4: Fluorescence intensity (IF), emission wavelength 660 nm, for P1 + 6 proteins with NR. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation

3.1.2 P2 (See Figure 5)

M0 adsorbs to the particle and possibly a conformational change occurs creating new hydrophobic binding sites for NR. M11 does not adsorb to the particle. M56 adsorbs to the particle. -2, +2 and +8 have greater affinity for the particles than for NR.

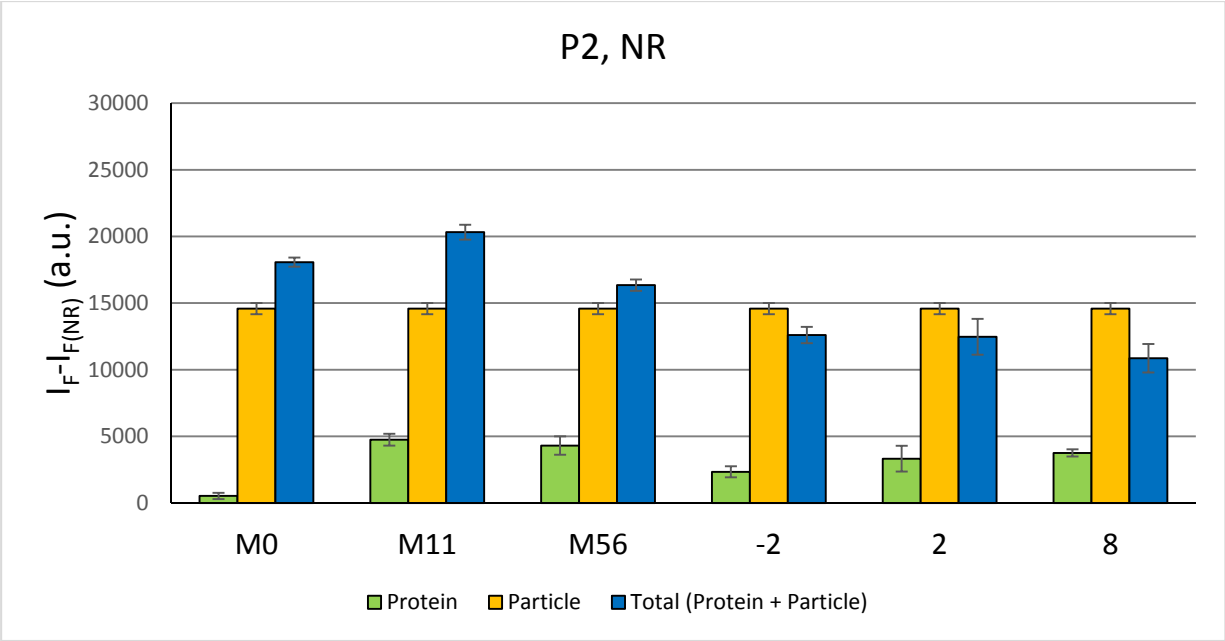

Figure 5: Fluorescence intensity (IF), emission wavelength 660 nm, for P2 + 6 proteins with NR. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation.

3.1.3 P3 (See Figure 6)

M0 does not adsorb to the particle. M11, M56 and +2 adsorbs to the particle. -2 and +8 have higher affinity for the particle than for NR.

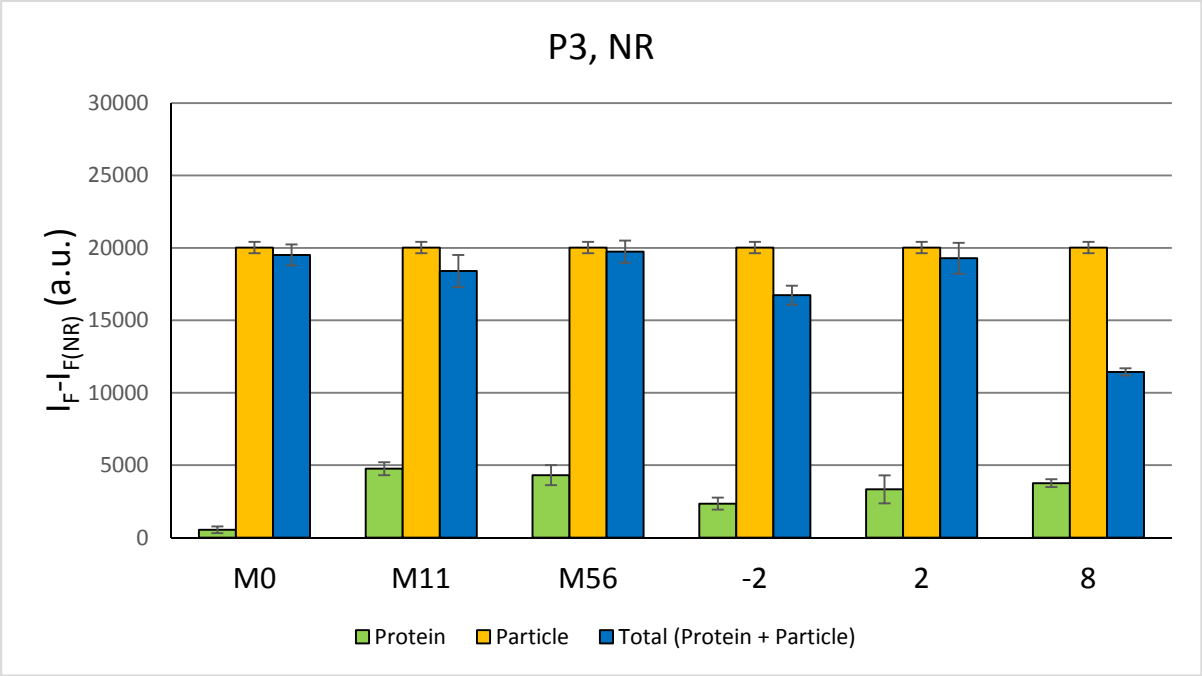

Figure 6: Fluorescence intensity (IF), emission wavelength 660 nm, for P3 + 6 proteins with NR. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation.

3.1.4 Raw data from full screening with NR

The raw data for the full screening with NR from time 0 – 10 h is shown in Figure 7. The $IF_{particle}$ for P1 decreases slightly over time. For P2 and P3 $IF_{particle}$ increases during the first hour and after two hours it stabilizes. $IF_{protein}$ increases during the first hour and after two hours it

stabilizes except for M0 which does not bind NR. IF $_{total}$ tends to increase during 1 – 2 hours and then stabilizes except for P3 + (+2) where it appears to be stable from the beginning.

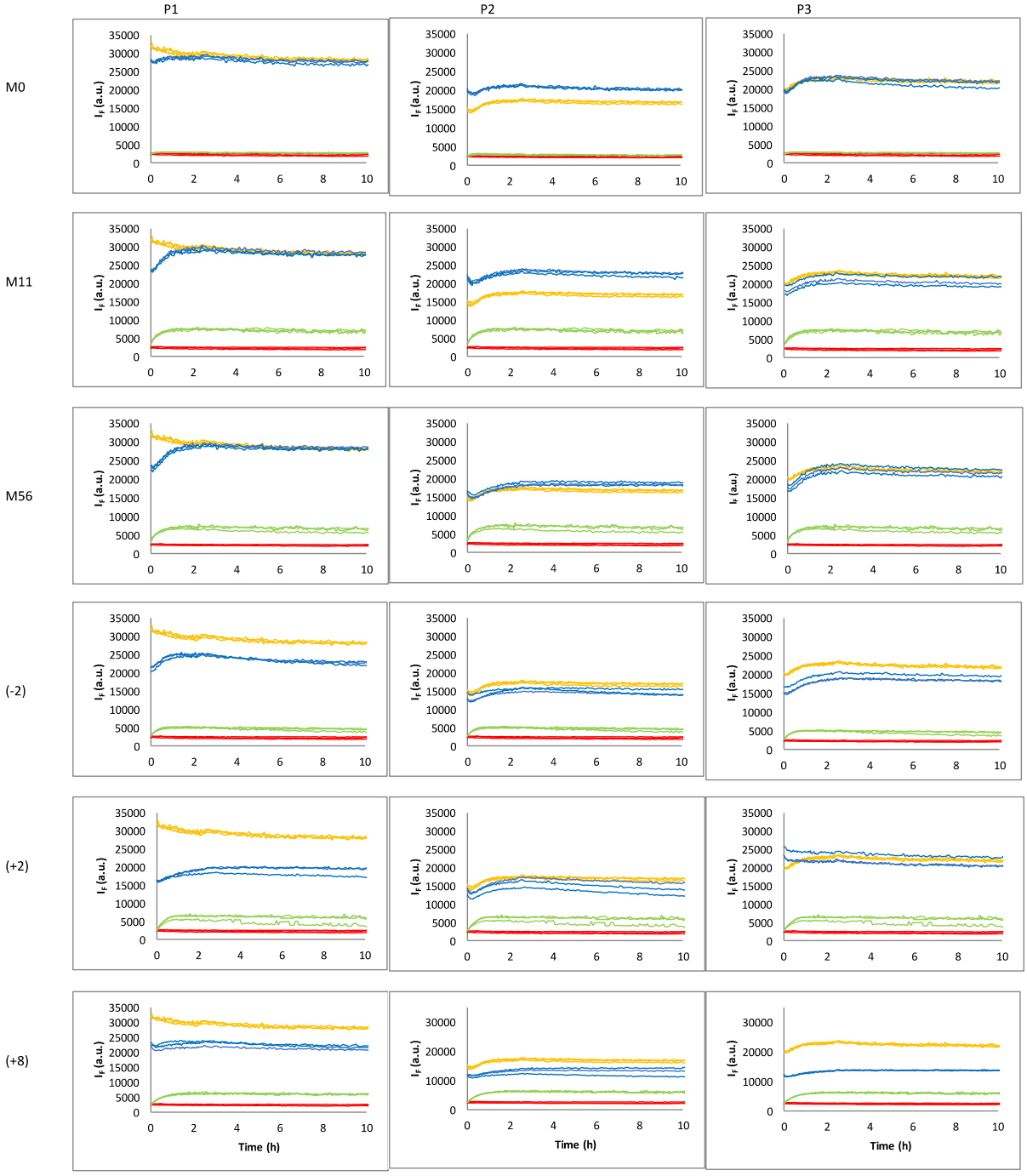

Figure 7: Raw data from full screening with NR, emission wavelength 660 nm, the 3 NPs and the 6 proteins. Red = NR, green = protein + NR, yellow = particle + NR and blue = protein + NR + particle.

3.2 Full screening with ANS

To detect interactions between the 3 NPs and the 6 proteins with ANS a plate plan was designed and fluorescence intensity was measured. (See plate plan 2 in appendix for specifics). ANS does not bind to the particle and therefore is only scenarios (a) considered.

3.2.1 P1 (See Figure 8)

M0 and +8 does not adsorb to the particle. M11 and M56 adsorbs to the particle. -2 and +2 adsorbs to the particle and possibly a conformational change creates new hydrophobic binding sites for ANS.

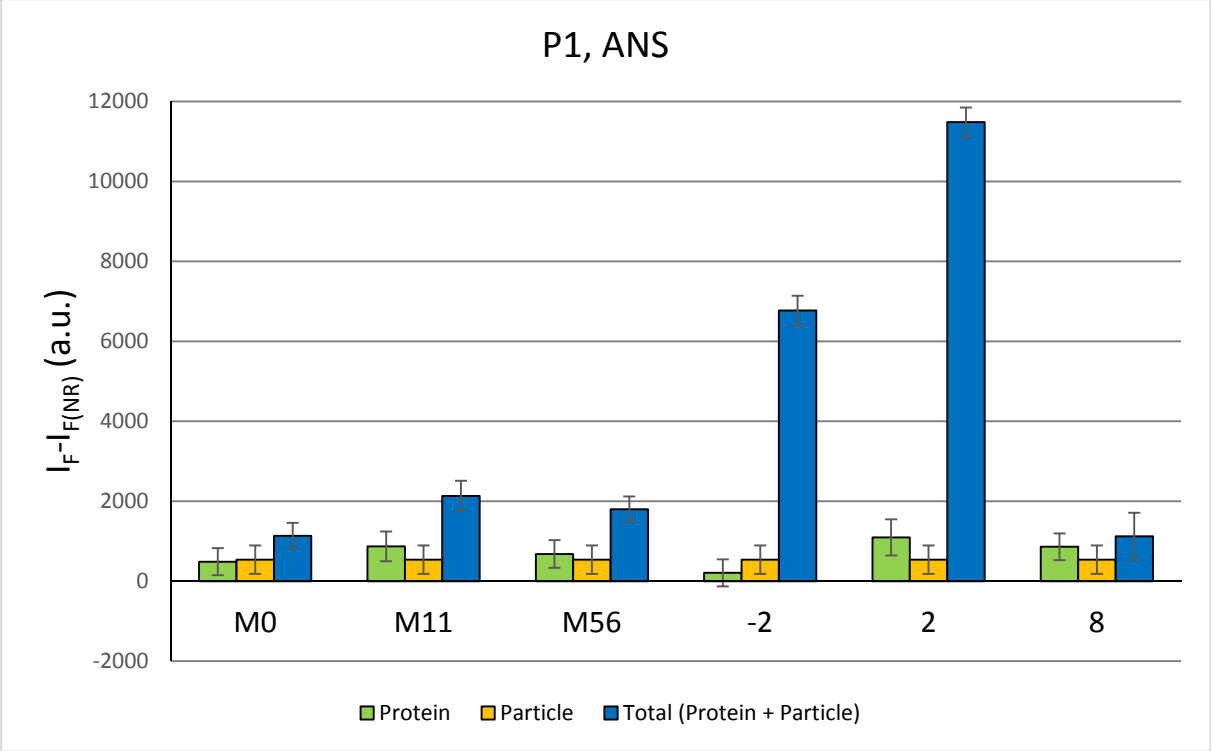

Figure 8: Fluorescence intensity (IF), emission wavelength 460 nm, for P1 + 6 proteins with ANS. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation.

3.2.2 P2 (See Figure 9)

M0, M11, M56 and +8 does not adsorb to the particle. -2 and +2 adsorbs to the particle and possibly a conformational change creates new hydrophobic binding sites for ANS

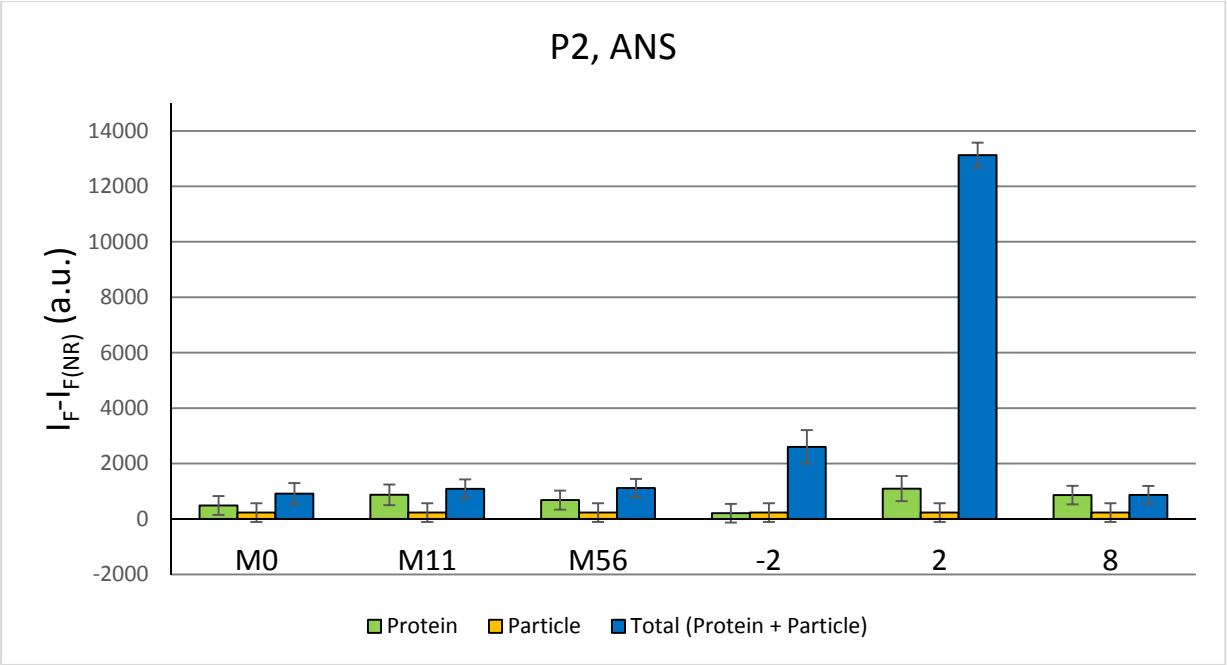

Figure 9: Fluorescence intensity (IF), emission wavelength 460 nm, for P2 + 6 proteins with ANS. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation.

3.2.3 P3 (See Figure 10)

M0, M11, M56 and +8 does not adsorb to the particle. -2 and +2 adsorbs to the particle and possibly undergoes a conformational change that creates new hydrophobic binding sites for ANS.

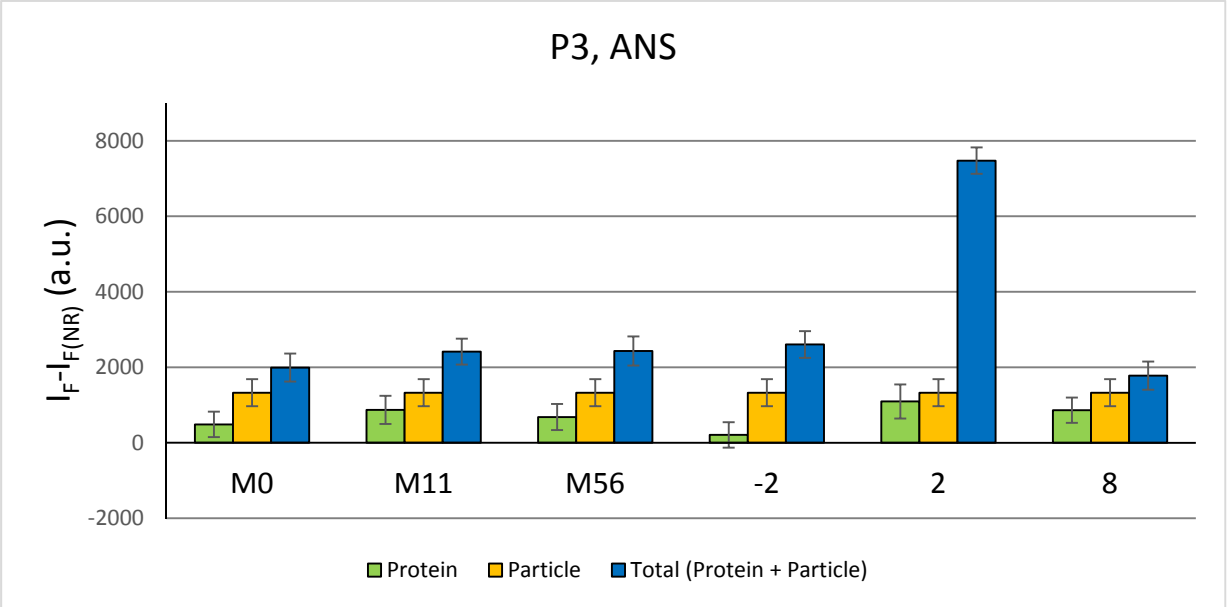

Figure 10: Fluorescence intensity (IF), emission wavelength 460 nm, for P3 + 6 proteins with ANS. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation

3.2.4 Raw data from full screening with ANS

The raw data for the full screening with ANS from time 0 – 10 h is shown in Figure 11. A majority of the samples tend to be stable directly from time 0 hour. The exception is (+2) + P1,

P2 and P3 as well as (-2) + P1 where the IF $_{total}$ increases rapidly the first 2 hours and then the increase declines. IF $_{total}$ for (-2) + P2 and P3 increases slightly over time.

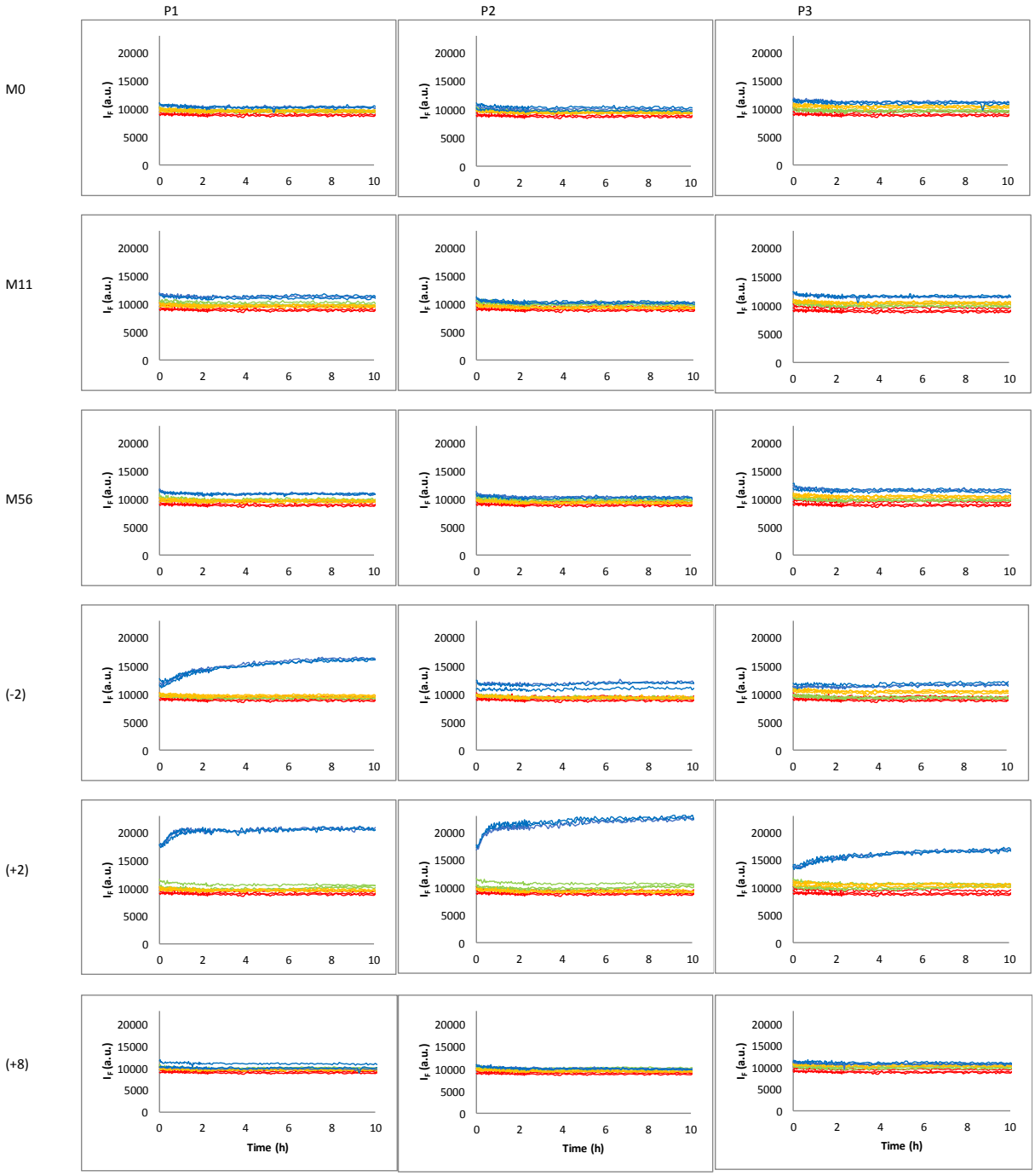

Figure 11: Raw data from full screening with ANS, emission wavelength 460 nm, the 3 NPs and the 6 proteins. Red = ANS, green = protein + ANS, yellow = particle + ANS and blue = protein + ANS + particle.

3.3 Hydrodynamic size

To determine the hydrodynamic size and the colloidal stability of the three different sized NPs DLS was used. (See plate plan 3 in appendix for specifics). After each full screening test with NR and ANS the plates were placed in the DLS plate reader to detect any aggregation in the samples. Table 1 and 2 shows the hydrodynamic diameter of NPs P1, P2 and P3 in HEPES

buffer as well as when the fluorophores and proteins were added. (See plate plan 1 and 2 in appendix for specifics). The only sample that seems to aggregate is P1 with +8.

Table 1: DLS data of the hydrodynamic diameter and standard deviation of NPs P1, P2 and P3 with NR and proteins. Calculated from the mean values of the 3 replicates, 16 hours after mixing the stock solutions.

	P1 Size (nm)	Std dev	P2 Size (nm)	Std dev	P3 Size (nm)	Std dev
In Hepes buffer	27,3	± 1,2	50,1	± 1,1	105,6	± 2,2
+ NR	27,7	± 1,3	52,9	± 1,2	100	± 2,9
With Calbindin D9k:						
M0	23,6	± 0,5	59,1	± 1,9	110,2	± 0,1
M11	26,3	± 4,6	60,3	± 0,6	112,6	± 2,0
M56	27	± 1,2	59,3	± 8,3	110,6	± 1,7
With Monelin:						
(-2)	25,6	± 0,9	50,2	± 8,8	111	± 1,8
(+2)	26,6	± 1,3	55,8	± 0,8	110,7	± 1,2
(+8)	~ 29,7 and 172,9	± 1,8	65,9	± 2,2	109,9	± 0,5

Table 2: DLS data of the hydrodynamic diameter and standard deviation of NPs P1, P2 and P3 with NR and proteins. Calculated from the mean values of the 3 replicates, 20 hours after mixing the stock solutions.

	P1 Size (nm)	Std dev	P2 Size (nm)	Std dev	P3 Size (nm)	Std dev
In Hepes buffer	27,3	± 1,2	50,1	± 1,1	105,6	± 2,2
+ ANS	24,6	± 1,2	52,9	± 4,3	105,1	± 1,1
With Calbindin D9k:						
M0	22,3	± 0,2	67,1	± 16,9	109,8	± 5,6
M11	22,7	± 2,2	62,5	± 11,9	105,7	± 2,3
M56	26,9	± 2,0	55,8	± 5,0	107,2	± 2,5
With Monelin:						
(-2)	25,8	± 0,5	38,7	± 11,1	105	± 0,1
(+2)	25,7	± 0,2	56,7	± 4,4	108,5	± 1,4
(+8)	26,4	± 0,1	53,3	± 2,9	105,1	± 1,8

3.4 Summary of screening data

Table 3 shows what scenarios the full screening data suggests and a combined analysis of the data if the proteins adsorb to the NPs.

Table 3: The scenarios the NR and ANS data indicates and a complete analysis of the full screening DLS data.

	P1 + NR	P1 + ANS		P2 + NR	P2 + ANS		P3 + NR	P3 + ANS	
Protein	Sce.[a]	Sce.[a]	C.A.[b]	Sce.[a]	Sce.[a]	C.A.[b]	Sce.[a]	Sce.[a]	C.A.[b]
With Calbindin D9k:									
M0	3b	3a	-	2b	3a	Ads[c]	3b	3a	-
M11	1b	2a	Ads[c]	3b	3a	-	1b	3a	Ads[c]
M56	1b	2a	Ads[c]	1b	3a	Ads[c]	1b	3a	Ads[c]
With Monelin:									
(-2)	1b	2a	Ads[c]	1b	2a	Ads[c]	1b	2a	Ads[c]
(+2)	1b	2a	Ads[c]	1b	2a	Ads[c]	1b	2a	Ads[c]
(+8)	1b	3a	Ads[c]	1b	3a	Ads[c]	1b	3a	Ads[c]

[a] Scenario number as presented in Figure 3.

[b] NR, ANS and DLS data Combined Analysis.

[c] The protein adsorbs to the NP.

3.5 NR concentration

To determine if different NR concentrations would result in different fluorescence results a plate plan was designed to test three different concentration of NR. (See plate plan 4 in appendix for specifics).

The three NR concentration resulted in different fluorescence intensity, higher concentrations leading to higher fluorescence intensity. 2 and 3 μM NR seems to have a negative effect on the $IF_{protein}$. (See figure 12, 13 and 14 in appendix).

3.6 Additional full screenings

Full screening with ANS and NR were additionally executed with similar results. The main difference between these screenings and the first ones was small changes in protein concentration and the stock solutions were left over night without proteins. The proteins were added right before the full screenings.

4. Discussion

4.1 Protein and NP interaction

4.1.1 M0 + P1

Both the NR and ANS full screening data indicates that M0 does not bind to P1. This is possibly because M0s total charge is negative -7 and P1s negatively charged COO^- groups repels each other. M0 does not bind ANS, maybe due to ANS negative charge.

4.1.2 M0 + P2

The NR data suggests that M0 binds to P2 which probably leads to a conformational change of M0 because IF_{total} is greater than $IF_{particle} + IF_{protein}$. The ANS data suggests no interaction but this could be because ANS is negatively charged and even if M0 binds to P2 and conformational changes creates new hydrophobic patches on the protein, the negative charges repel each other. Nasir et al. (2015) showed similar results when using the same method, protein and NP. The fact that M0 interacts with P2 but not with P1 and P3 could be because they are produced by two different companies. P2 could have fewer COO^- groups and M0 is therefore not repelled.

4.1.3 M0 + P3

NR and ANS data indicates no interaction between M0 and P3.

4.1.4 M11 + P1

Both NR and ANS data suggests that M11 binds to P1. This could be because M11s total charge is -4 and might therefore bind to P1s hydrophobic patches without getting repelled by P1 COO^- groups. The ANS data also shows a possible conformational change of M11 because IF_{total} is greater than $IF_{particle} + IF_{protein}$.

4.1.5 M11 + P2

NR and ANS data indicates no interaction between M11 and P2 because IF_{total} is equal to $IF_{particle} + IF_{protein}$. This is probably not due to the greater size of P2 but because P2 is purchased from a different company than P1 and P3 and might therefore not be exactly the same. P2 has probably fewer COO^- groups and the total charge of M11 is -4 which could be the reason why M0 (-7) does interact with P2 while M11 (-4) does not.

4.1.6 M11 + P3

The NR data suggests that M11 binds to P3 because $IF_{particle} + IF_{protein}$ is greater than IF_{total}.

4.1.7 M56 + P1

The NR data indicates interaction between M56 and P1 and the ANS data shows that a conformational change could have occurred because IF_{total} is greater than $IF_{particle} + IF_{protein}$. M56 (-7) is less stable than M0 and could perhaps alter its shape to interact with the P1

4.1.8 M56 + P2

The ANS data shows no interactions but the NR data strongly indicates that M56 binds to P2.

4.1.9 M56 + P3

The NR data suggests interaction between M56 and P3 because $IF_{particle} + IF_{protein}$ is greater than IF_{total}. From the ANS data it is difficult to make any assumptions.

4.1.10 -2 + P1

Both NR and ANS data indicates that -2 interacts with P1. The ANS data strongly suggests a conformational change since IF_{total} is greater than $IF_{particle} + IF_{protein}$. The raw data with ANS shows that the IF_{total} increases over time thus similarly indicating a conformational change of -2.

4.1.11 -2 + P2

The NR data suggests interaction between -2 and P2. The ANS data indicates a conformational change but not as strongly as for -2 + P1 and could therefore depend of the NPs size.

4.1.12 -2 + P3

The NR data indicates that -2 binds to P3. The ANS data suggests conformational changes of -2 but not as strongly as -2 + P2 and therefore indicating that the conformational change is dependent on the size of the NP.

4.1.13 +2 + P1

The NR data suggests that +2 binds to P1 and the ANS data strongly indicates a conformational change of +2 because IF_{total} is greater than $IF_{particle} + IF_{protein}$. The ANS raw data shows that the conformational change or binding occurs rapidly because of the fast increase of IF_{total}. The binding of +2 to P1 could possibly be because +2s total charge is positive and attracts to P1s COO^- groups.

4.1.14 +2 + P2

Both NR and ANS data indicates interaction between +2 and P2. The ANS data and raw data from ANS strongly suggests a conformational change or binding occurring rapidly.

4.1.15 +2 + P3

The NR and ANS data suggests that +2 interacts with P3 and undergoes a conformational change. The ANS raw data indicates the conformational change or the binding occurs slower than for P1 and P2 suggesting that interaction is dependent on the NPs size.

4.1.16 +8 +P1

The NR data strongly indicates interaction between +8 and P1 while the ANS suggests no interaction. This might be because +8 binds to P1 but no conformational change occurs leading to no new hydrophobic binding sites on +8 where ANS can bind to.

4.1.17 +8 + P2

Similarly to +8 + P1 the NR data suggests interaction but no conformational change.

4.1.18 +8 + P3

As for +8 + P1 and P2 the NR data indicates interaction but no conformational change of +8.

The possible conformational change of the proteins when adsorbed to the NPs could also be because proteins on the NPs interacts with other proteins, forming a second layer of proteins on the NP and therefore, creating new hydrophobic patches. To determine what kind of interactions occurs methods as circular dichroism (CD) (Bardhan et al., 2009) and transmission electron microscopy (TEM) (Reymond-Laruinaz et al., 2016) could be used.

4.2 DLS data

The results from the DLS data shows that all samples except +8 + P1 did not aggregate. This could be because +8 interacted with P1 and a possible conformational change occurs. This change in the proteins 3D structure could have led to the proteins and NPs aggregation. The DLS data is a great way to notice any substantial aggregation and thus the NPs colloidal stability but it is also very sensitive to bigger particles e.g. dust particles or undissolved protein. Bigger particles interrupt the smaller particles scattered light and therefore alters the result. This sensitivity is easily seen in Table 1 and 2 where particles hydrodynamic diameter alters ± 5 - 10 nm. To get more accurate data more homogenous samples are needed.

4.3 NR concentration

Higher concentration of NR in the samples led to higher fluorescence intensity. This could be due to that the particles were not fully covered of NR. However, the concentration 1 μM NR was calculated to fully cover the NPs. Another explanation could be that NR interacts with already bound NR to the particles and thus creating more layers of NR around the particle. 2 and 3 μM NRs negative effect on $IF_{protein}$ could be because the proteins interact in some way with NR that leads to less emission from NR but this seems only to occur when NR concentrations are greater than 1 μM.

4.4 Additional full screenings

The results from the additional full screenings compared to first full screenings results suggests that the results are reproducible.

5. Conclusion

We have shown that hydrophobicity and charge of proteins along with NPs size are factors that determines whether proteins and NPs interact. The method used in this report is a great system to study general interactions and to find interesting interactions to later study in greater detail. To do so other methods need to be considered e.g. circular dichroism, transmission electron microscopy or infrared spectroscopy. Due to increased usage and interest in nanoparticles, the possible hazardous effect is extremely important to study and here we have added an important piece to the protein-nanoparticle puzzle.

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7. Appendix

Plate plan 1

Concentrations in each well:

NR = 1 μM.

P1, P2 and P3 = 0.1 mg/ml.

Calbindin D_{9k}:

M0 = 0.1 mg/ml.

M11 = 0.1023 mg/ml.

M56 = 0.1 mg/ml.

Monellin:

-2 = 0.094 mg/ml.

+2 = 0.1 mg/ml.

+8 = 0.0243 mg/ml.

Table 4: Plate plan 1 design for full screening test with NR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NR	NR	NR	NR+M0	NR+M0	NR+M0	NR+M11	NR+M11	NR+M11	NR+M56	NR+M56	NR+M56
B	P1+NR	P1+NR	P1+NR	P1+NR+M0	P1+NR+M0	P1+NR+M0	P1+NR+M11	P1+NR+M11	P1+NR+M11	P1+NR+M56	P1+NR+M56	P1+NR+M56
C	P2+NR	P2+NR	P2+NR	P2+NR+M0	P2+NR+M0	P2+NR+M0	P2+NR+M11	P2+NR+M11	P2+NR+M11	P2+NR+M56	P2+NR+M56	P2+NR+M56
D	P3+NR	P3+NR	P3+NR	P3+NR+M0	P3+NR+M0	P3+NR+M0	P3+NR+M11	P3+NR+M11	P3+NR+M11	P3+NR+M56	P3+NR+M56	P3+NR+M56
E				NR+(-2)	NR+(-2)	NR+(-2)	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+8)	NR+(+8)	NR+(+8)
F				P1+NR+(-2)	P1+NR+(-2)	P1+NR+(-2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+8)	P1+NR+(+8)	P1+NR+(+8)
G				P2+NR+(-2)	P2+NR+(-2)	P2+NR+(-2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+8)	P2+NR+(+8)	P2+NR+(+8)
H				P3+NR+(-2)	P3+NR+(-2)	P3+NR+(-2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+8)	P3+NR+(+8)	P3+NR+(+8)

Plate plan 2

Concentrations in each well:

NR = 1 μM.

P1, P2 and P3 = 0.1 mg/ml.

Calbindin D_{9k}:

M0 = 0.1 mg/ml.

M11 = 0.1 mg/ml.

M56 = 0.1 mg/ml.

Monellin:

-2 = 0.14 mg/ml.

+2 = 0.1 mg/ml.

+8 = 0.0243 mg/ml.

Table 5: Plate plan 2 design for full screening test with ANS

	1	2	3	4	5	6	7	8	9	10	11	12
A	ANS	ANS	ANS	ANS+M0	ANS+M0	ANS+M0	ANS+M11	ANS+M11	ANS+M11	ANS+M56	ANS+M56	ANS+M56
B	P1+ANS	P1+ANS	P1+ANS	P1+ANS+M0	P1+ANS+M0	P1+ANS+M0	P1+ANS+M1	P1+ANS+M1	P1+ANS+M1	P1+ANS+M5	P1+ANS+M5	P1+ANS+M5
C	P2+ANS	P2+ANS	P2+ANS	P2+ANS+M0	P2+ANS+M0	P2+ANS+M0	P2+ANS+M1	P2+ANS+M1	P2+ANS+M1	P2+ANS+M5	P2+ANS+M5	P2+ANS+M5
D	P3+ANS	P3+ANS	P3+ANS	P3+ANS+M0	P3+ANS+M0	P3+ANS+M0	P3+ANS+M1	P3+ANS+M1	P3+ANS+M1	P3+ANS+M5	P3+ANS+M5	P3+ANS+M5
E				ANS+(-2)	ANS+(-2)	ANS+(-2)	ANS+(+2)	ANS+(+2)	ANS+(+2)	ANS+(+8)	ANS+(+8)	ANS+(+8)
F				P1+ANS+(-2)	P1+ANS+(-2)	P1+ANS+(-2)	P1+ANS+(+2	P1+ANS+(+2	P1+ANS+(+2	P1+ANS+(+8	P1+ANS+(+8	P1+ANS+(+8
G				P2+ANS+(-2)	P2+ANS+(-2)	P2+ANS+(-2)	P2+ANS+(+2	P2+ANS+(+2	P2+ANS+(+2	P2+ANS+(+8	P2+ANS+(+8	P2+ANS+(+8
H				P3+ANS+(-2)	P3+ANS+(-2)	P3+ANS+(-2)	P3+ANS+(+2	P3+ANS+(+2	P3+ANS+(+2	P3+ANS+(+8	P3+ANS+(+8	P3+ANS+(+8

Plate plan 3

Concentrations in each well.

P1 = 0.065 mg/ml.

P2 = 0.155 mg/ml.

P3 = 1.18 mg/ml.

Table 6: Plate plan 3 design for measuring the hydrodynamic diameter and colloidal stability with DLS.

	1	2	3
A	Water+P1	Water+P2	Water+P3
B	Hepes+P1	Hepes+P2	Hepes+P3

Plate plan 4

Concentrations in each well:

Light green = 1 μM NR, light blue 2 μM NR and dark blue = 3 μM NR.

P1, P2 and P3 = 0.1 mg/ml.

Monellin +2 = 0.1 mg/ml.

Table 7: Plate plan 4 design for NR concentration determination.

	1	2	3	4	5	6	7	8	9
A	NR	NR	NR	NR	NR	NR	NR	NR	NR
B	P1+NR	P1+NR	P1+NR	P1+NR	P1+NR	P1+NR	P1+NR	P1+NR	P1+NR
C	P2+NR	P2+NR	P2+NR	P2+NR	P2+NR	P2+NR	P2+NR	P2+NR	P2+NR
D	P3+NR	P3+NR	P3+NR	P3+NR	P3+NR	P3+NR	P3+NR	P3+NR	P3+NR
E	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+2)	NR+(+2)
F	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)	P1+NR+(+2)
G	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)	P2+NR+(+2)
H	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)	P3+NR+(+2)

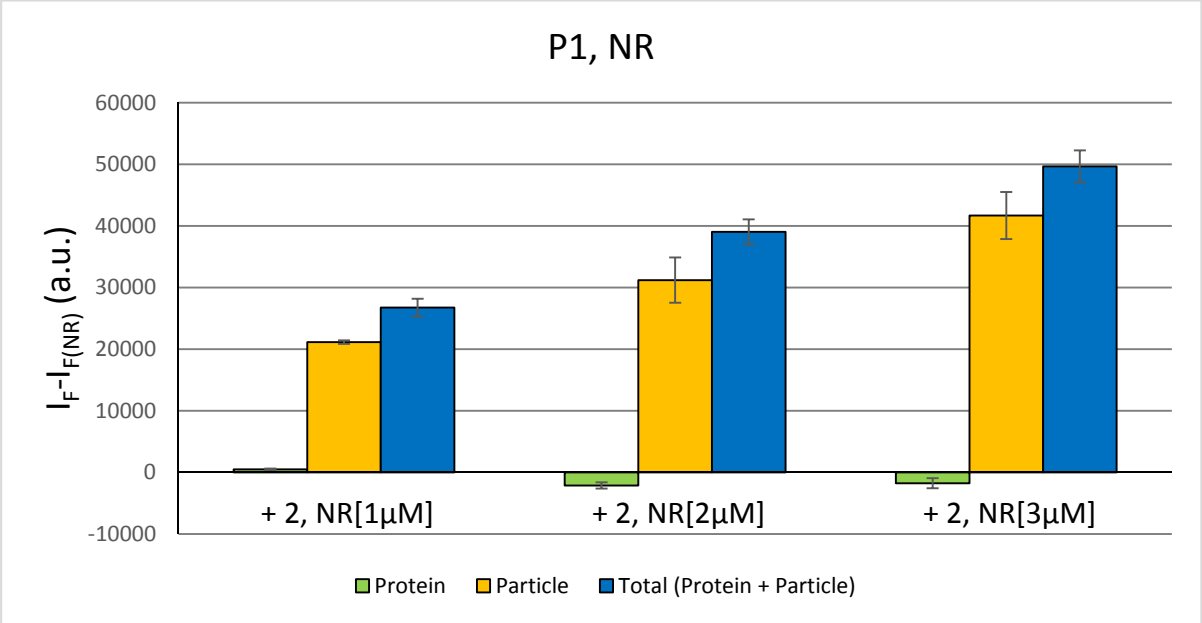

Figure 12: Fluorescence intensity (IF), emission wavelength 590 nm, for P1 + (+2) with NR concentrations, 1, 2 and 3 µM. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard deviation.

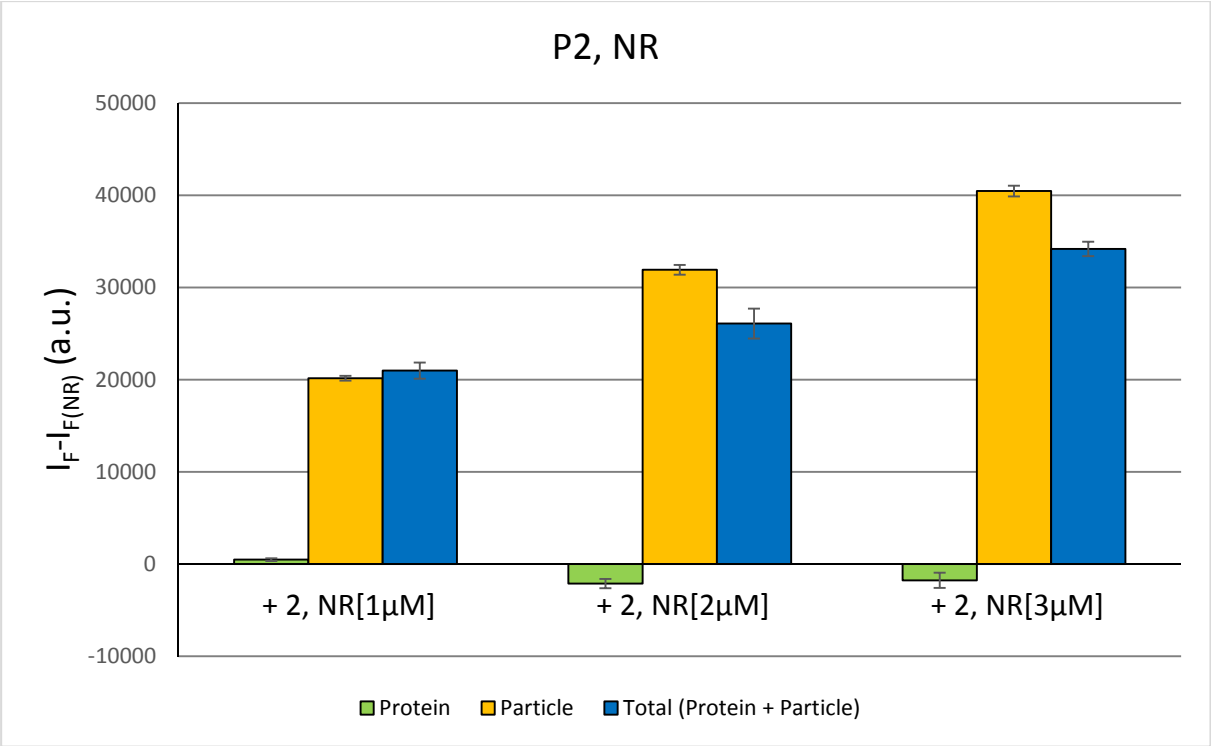

Figure 13: Fluorescence intensity (IF), emission wavelength 590 nm, for P2 + (+2) with NR concentrations, 1, 2 and 3 µM. Bars represent the mean value from three replicates, 7 hours after mixing the

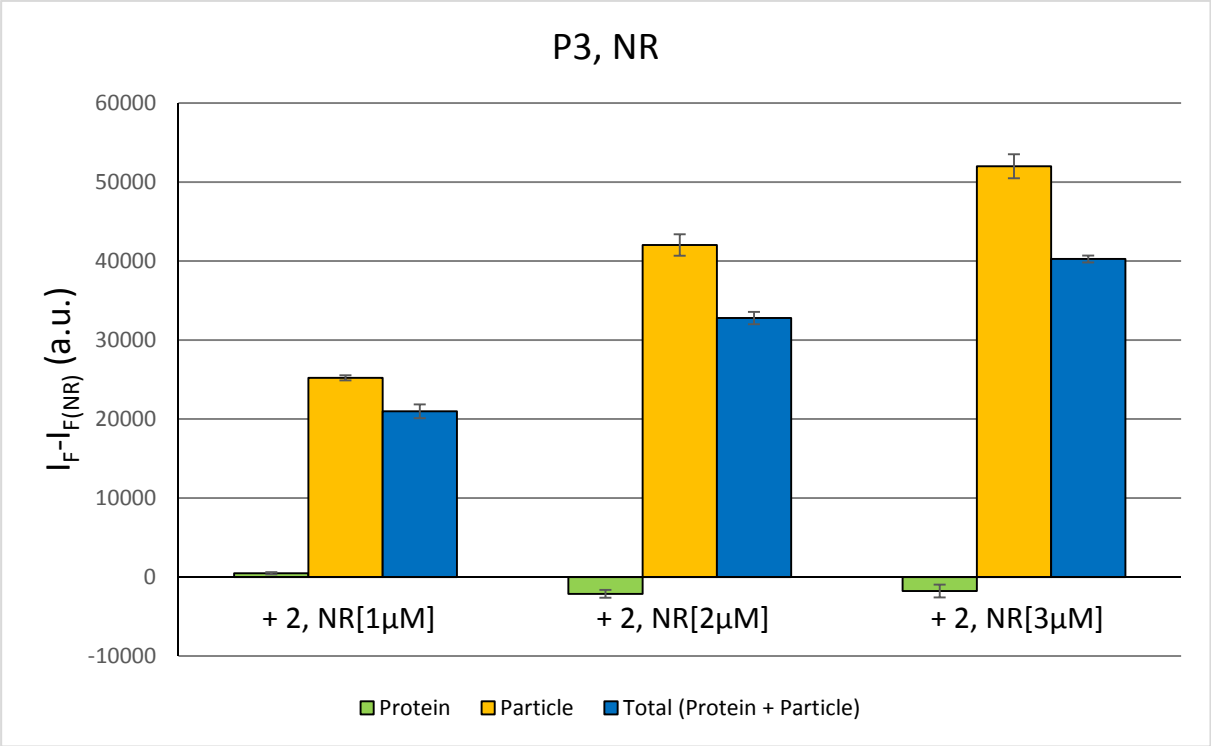

Figure 14: Fluorescence intensity (IF), emission wavelength 590 nm, for P3 + (+2) with NR concentrations, 1, 2 and 3 µM. Bars represent the mean value from three replicates, 7 hours after mixing the stock solutions and error bars are their corresponding standard

Risk assessment:
Dimethyl Sulfoxide (DMSO):
H227 Combustible liquid.
H315 Causes skin irritation.
H319 Causes serious eye irritation.

P210 Keep away from heat / sparks / open flames / hot surfaces - No smoking.
P264 Wash thoroughly after handling.
P280 Wear protective gloves / eye protection / face protection.
P302+352 IF ON SKIN: Wash with plenty of soap and water.
P305+351+338 IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing.
P337+313 If eye irritation persists: Get medical advice / attention.
P362 Take off contaminated clothing and wash before reuse.
P403+235 Store in a well ventilated place. Keep cool.
P501 Dispose of contents / container in accordance with local / national regulations.