Forming the protein corona on nanoparticles

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Abstract

Nanomedicine is a growing field with great potential. When nanoparticles (NPs) come in contact with biological fluids a protein corona is formed on the surface. The protein corona has an effect on the pharmacokinetics and biological response. This might in turn lead to disadvantageous drug delivery and needs to be addressed when using NPs as drug delivery tools. How to build the protein corona and what effect different proteins have is a live research topic. In this study we investigate whether adding the same volume of polystyrene-COOH NPs, but in different aliquots, into a solution of immunoglobulin G (IgG) and other proteins has an effect of protein corona formation and NP aggregation. The aggregation formation is studied with the help of dynamic light scattering and absorbance measurements. We observed that that higher protein concentration did not lead to higher aggregation. The highest aggregation was found to form at a concentration of 0.1 mg/ml IgG. When the NPs were added in different aliquots the final aggregation was found to be larger compared to a single addition alone. Comments about weakness, improvements and potential use of the study are offered. An outlook on the future field is also briefly discussed.

1. Introduction

One definition of nanoparticles is that they need to have at least one external surface with a dimension ranging from 1-100 nm or having a greater surface area than $60 m^2/cm^3$. (Rauscher et al., 2013).

The use of nanoparticles (NPs) has increased greatly due to their various properties. They are already used in multiple industries, including the cosmetic and food industries (Calzolai et al., 2012; Raj et al., 2012). In recent years the interest for nanomedicine has also increased, especially drug delivery (Emerich and Thanos, 2006; Schroeder et al., 2007). This interest has grown due to the potential of superior solubility, biodistribution and pharmacokinetics and thus more targeted drug delivery. (Riehemann et al., 2009) NPs might however cause negative effects on the cell depending on multiple factors, including NPs surface, size and composition. (Manke et al., 2013) It is thus important to build the NPs in an exact manner for use in medicine to improve efficiency and side-effects profile.

It is widely known that macromolecules bind to NPs in serum and a protein corona is formed. (Walkey and Chan, 2012) The corona can be divided into two different parts, the hard corona which has tightly bound proteins with high affinity for the NP and the soft corona which has loosely bound proteins with low affinity for the NP (Milani et al., 2012). The corona composition is dependent on multiple factors including size, surface properties and whether the patient is in a diseased state or not (Caracciolo et al., 2014; Lundqvist et al., 2008). Depending on what makes up the corona the biological

response might change. It has previously been shown that the corona is quickly formed with serum albumin being the first major protein binding for certain NPs. However if the NP corona is exposed to proteins over longer intervals of time, other proteins start to associate and serum albumin starts to dissociate. (Dell'Orco et al., 2010) Because the corona is the first thing the cells notice it is the corona that decides the biological response. This further complicates the use of nanoparticles for drug delivery or any other use in biological tissues.

For example, when carbon nanotubes bind serum protein the cytotoxicity is greatly reduced (Ge et al., 2011) and when transferrin binds FePt NPs the uptake by HeLa cells is greatly reduced. (Jiang et al., 2010) Changes like these might render the NPs useless as drug delivery tools (Salvati et al., 2013). The corona also changes until it reaches equilibrium, but will change again if the environment changes. (Lundqvist et al., 2011) There are however methods to reduce the adsorption, for example with polyethylene glycol (PEG). PEG is able to reduce protein adsorption, but fails to stop it completely (Gref et al., 2000). Proteins bound to the corona might also change conformations which might expose new parts of the protein that are usually inactive (Roach et al., 2006; Shemetov et al., 2012).

IgG is composed of four chains in total, two heavy chains (50 kDa each) and two light chains (25 kDa each). It is the most common antibody in the serum making up 10-20% of the total serum protein. Due to the sulfide bonds IgG gets a Y-shaped conformation, with two Fab domains at the top and an Fc region at the bottom. The Fab domain binds antigens and tags them for phagocytosis while Fc region is the part of the IgG molecule that binds to the Fc-receptors and initiates phagocytosis.(Biburger et al., 2014)

For NPs to be used as a drug delivery tool, the first step would be to control the corona formation. The aim of this study is to investigate different factors affecting protein corona formation with a focus on polystyrene nanoparticles with a modified carboxyl group surface (PS-COOH) and IgG. The concentration of IgG where aggregation occurs the most is determined. Different aliquots are being investigated to determine whether they have an effect on protein corona formation.

2. Material and methods.

2.1 NP and Protein

Polystyrene NPs surface modified with a carboxyl group (PS-COOH) were purchased from Bang Laboratories, Inc. (Fisher, IN, USA). The NPs were used as received. The mean radius of PS-COOH was determined by the use of dynamic light scattering (DLS). PS-COOH were diluted to 0.5 mg/ml with water before all measurements. IgG were purchased from Lee Biosolutions (St. Louis, MO, USA). IgG was diluted with water to 0.1 mg/ml before all measurements.

2.2 Protein purification

IgG was purified by using dialysis with a molecular weight cut-off of 3500 Da in water. The water was changed on a daily basis for three days until the protein was transferred to a new container and frozen.

2.3 Absorbance measurements

Absorbance measurements were performed using Probe Drum (Probation Labs, Sweden). The NP solution was titrated in the protein solution using Probe Drum. Spectrophotometric data was obtained and then analyzed. A total of 100 µl NP solution was titrated into 900 µl protein solution (0.1 mg/ml) in different volume additions (see Probe Drum settings in appendix for specific settings).

2.4 DLS measurements

The hydrodynamic radius of the NP-protein aggregates and NP alone were determined by DLS using DynaPro Platereader-II (Wyatt Technology, CA, USA). Measurements were taken after the IgG mixture had been incubated with PS-COOH for 20 minutes at 25 °C. Two different protocols were performed (see DLS protocol 1 & 2 and table 1 & 2 in appendix for specifics). In the first test the NP concentration tested varied 0.01 to 0.5 mg/ml and the IgG concentration varied from 0.005 and 3 mg/ml. The mean of three measurements was used. In the second test IgG concentration varied from 0.005 to 0.05 mg/ml and NP concentration from 0.05 to 0.5 mg/ml. The mean of four measurements were used.

3. Results

3.1 NP Measurement

The hydrodynamic radius of PS-COOH was determined to be approximately 16 nm with a standard deviation of 0.5 nm by the use of DLS. Measurements were taken in quadruplets. The measurements were not dependent on PS-COOH concentration, suggesting no significant self-aggregation occurred.

3.2 One addition of NP

We first evaluated which IgG concentration resulted in the highest absorbance (see fig 1 & 2) with additions of 0.5 mg/ml PS-COOH. Prior testing with 0.1 mg/ml PS-COOH resulted in low absorbance, thus the NP concentration was increased. The maximum absorbance was measured as 0.20 Au at 0.1 mg/ml IgG with an average of 0.18 Au. Due to lack of robust data the hydrodynamic radius could not reliable be determined for single addition of PS-COOH to different IgG concentrations. The trend did however show that hydrodynamic radius peaked at 0.1 mg/ml IgG.

Initial results of a mixture between IgG and serum albumin with added PS-COOH showed much lower absorbance, suggesting, due to its smaller size, serum albumin and IgG competing for surface binding to PS-COOH (data not shown).

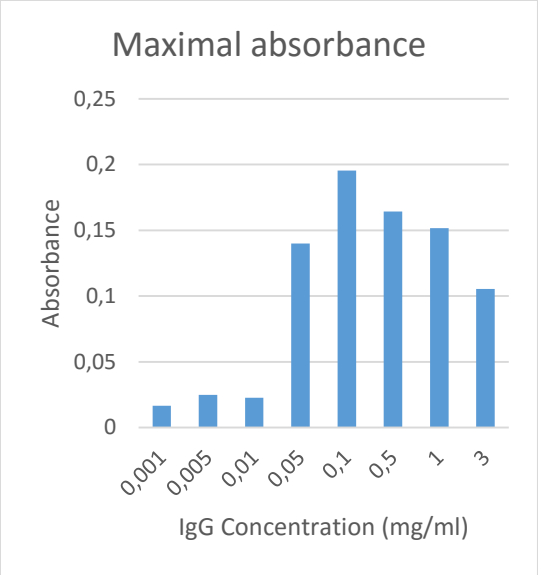

Figure 1. Maximal absorbance after a single addition of 100 µl 0.5 mg/ml PS-COOH to IgG.

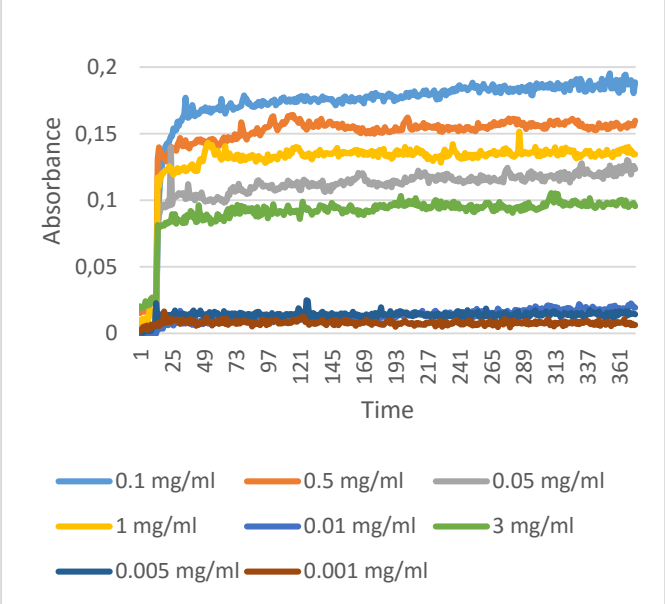

Figure 2. Absorbance of IgG-PS-COOH aggregates before and after a single addition of 100 µl 0.5 mg/ml PS-COOH as a function of time

3.3 Multiple additions of NP.

Following the initial measurements, additions of different aliquots with a total of 100 µl 0.5 mg/ml PS-COOH was measured. Fig 3a shows the addition in 20 µl aliquots, a clear equilibrium is achieved before the addition of more NPs. The final absorbance was approximately 0.21 Au.

Fig 3b shows the addition in 5 µl aliquots, the equilibrium is achieved clearly achieved in the start of the experiment, but as the time goes on the equilibrium is not as clear. Towards the end the absorbance decreases for unknown reasons. The final absorbance is approximately 0.20 Au.

Fig 3c shows the addition in 33.33 µl aliquots, the equilibrium after each addition is clearly achieved. The final absorbance is approximately 0.26 Au.

Fig 3d shows the addition in 50 µl aliquots, the equilibrium after each addition is clearly achieved. The final absorbance is approximately 0.25 Au.

The addition in 1 µl aliquots achieved equilibrium in the beginning but not clearly towards the end. Towards the end the absorbance decreased.

All measurements resulted in a higher average final absorbance (see fig 3e). The hydrodynamic radius could not reliably be determined for multiple additions of PS-COOH to different IgG concentrations. The trend that could be seen did however show that multiple additions of PS-COOH led to a higher final hydrodynamic radius.

3.4 DLS results from multiple additions vs one.

The hydrodynamic radius showed a trend towards increasing when aliquots were used. There was however data points missing, but averages were used to decrease the error. No statistical test was performed.

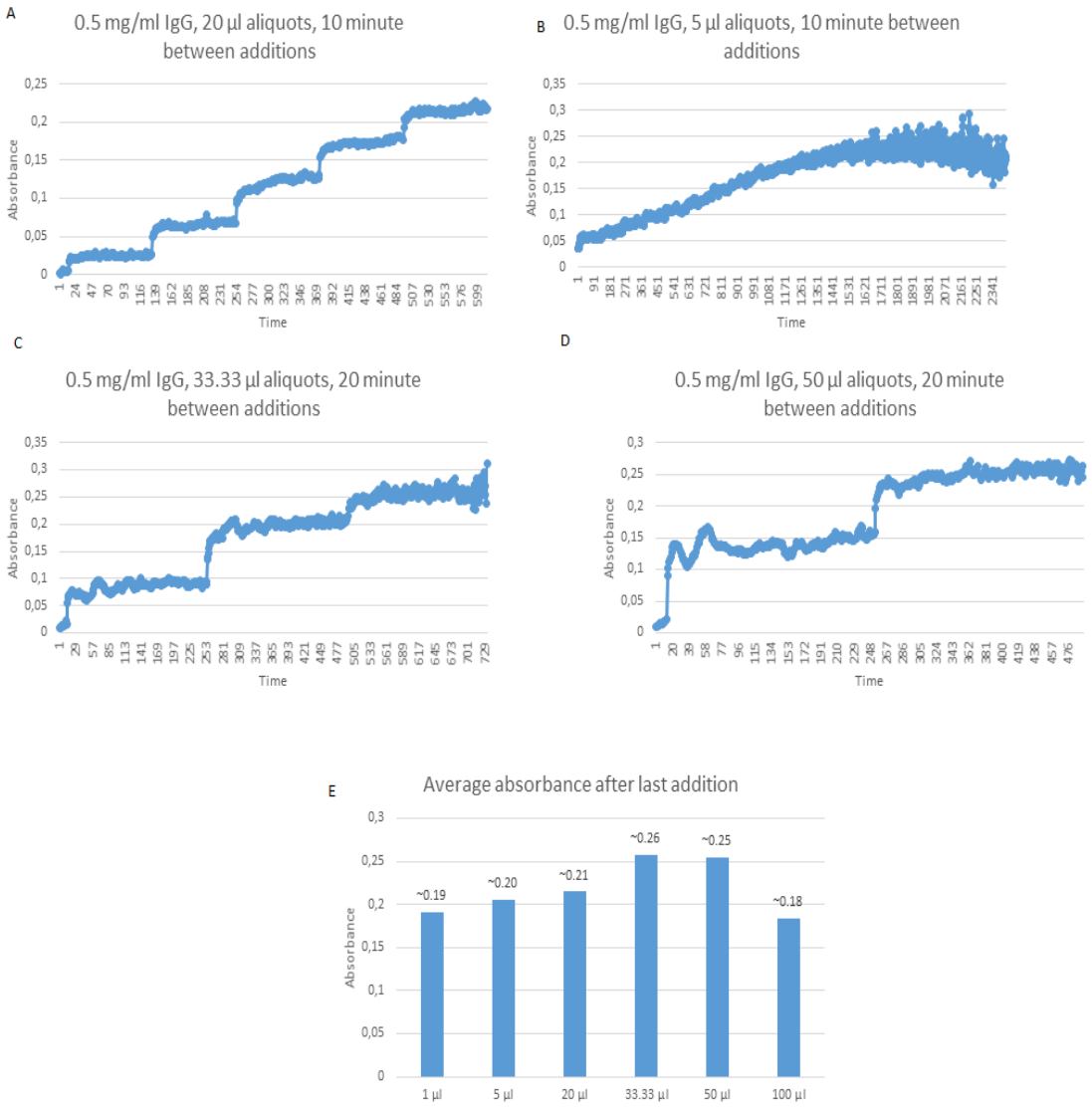

Figure 3. A) PS-COOH was titrated in 20 µl aliquots. B) PS-COOH was titrated in 5 µl aliquots C) PS-COOH was titrated in 33.33 µl aliquots D) PS-COOH was titrated in 50 µl aliquots E) The average absorbance after the last addition of PS-COOH for all the different aliquots.

4. Discussion

These results suggest that adding the same volume in different aliquots has an effect on the protein corona formation (fig 3E). The results show that smaller aliquots do lead to a higher final absorbance, but they did however not lead to maximum absorbance.

The cause of the decrease in absorbance of the 1 and 5 µl aliquots should be investigated. Testing multiple volume additions and examining at which leads to the highest absorbance should be done in the future. Based on this and previous work we know that we are able to at least partially control the

protein corona formation. (Docter et al., 2015) As the corona is the major decider of biological response this is the first step on creating specialized NPs. (Yan et al., 2013) The use of IgG in the corona might help promote phagocytosis by macrophages in the reticuloendothelial system and thus lead to a more targeted NP with a shorter half-life. (Owens and Peppas, 2006) In contrast to this, serum albumin has been shown to increase the half-life greatly. (Ogawara et al., 2004) Being able to control the half-life reduces the risk of blood accumulation to toxic levels. It can also help to increase the level of drugs in the blood over longer period of times so they stay active longer and re-dosing is not needed.

IgG and serum albumin competes for binding to the surface of PS-COOH as previously shown with other NPs. (Dell'Orco et al., 2010)

To determine the size of the particles in a more reliable manner more techniques should be used. This has been shown in previous studies where more data can be obtained by using multiple methods. (Bootz et al., 2004) While DLS is the most common and most used method, it has drawbacks. DLS preparation is important as contaminations, e.g. dust particles, might skew the results. Related to this is also that DLS requires the particle size distribution be fairly narrow for it to work. This is not a major problem in this case, but depending on the NP preparation it might be. Another drawback is that if the concentration that is measured is too high it will result in a lower hydrodynamic radius due to different scatter patterns and because bigger particles scatter light with more intensity compared to small.

Electron microscopes are also used to characterize NPs and to obtain a 2D-image of the NPs. This method can both handle higher and lower concentration of NPs compared to DLS. It does however still need a large sample size to get a descriptive picture and will not be able to give the thickness of the layers. One problem is however the preparation of the samples. If TEM is used the samples usually need to be dried which might result in the loss of the protein corona. This problem has however been overcome with newer techniques. (Thiberge et al., 2004) The preparation of the samples needs to be done correctly to get an accurate picture. Using different preparations might result in incorrect results. Depending on the resolution it might also be hard to distinguish individual NPs from other individual NPs. It is also time consuming as the particles need to be manually counted to get an exact result.

Disc centrifugation is another widely used technique to decide NP size. The size is determined by the use of settling velocity in a liquid with known density and viscosity. It is also able to detect changes in surface properties and is able to determine resolution in the low Ångström. A drawback is however that the run time cannot be too long as the density gradient degrades. But by combining multiple techniques the errors can be reduced and the amount of useful data is increased.

First and foremost this study needs to be replicated. To ensure accurate results, triplets should be used. An inherent weakness of the present study is the lack of replication during the experiment. Replications were only done when we were unsure if the data was correct. Furthermore testing different titrants and solutions is of interest. These tests

include testing multiple proteins in a solution and titrating PS-COOH, and testing different proteins as titrants. It would also be of interest to add PEG towards the end of the NP additions and then add extra proteins to see if PEG is able to stabilize the corona. Also adding the PS-COOH with protein corona into serum would be worth studying, with and without PEG. Further studies on which proteins regulate the cellular uptake might make it possible to form more specific protein coronas. Changing the surface area of the NP might also change the response as previously shown. (Monopoli et al., 2011)

5. Conclusion

As the NP field grows it is expected to see more research on the protein corona and what factors has an effect on its formation. Even though a lot has been uncovered the field is still in its infancy. This research has uncovered a new factor concerning protein corona formation, adding the NPs in different aliquots. This research is basic research on protein corona formation and holds potential to be used as in the future when forming protein coronas.

More research is however needed to confirm and expand on this subject. A future application of controlling the protein corona could lead to better and safer drug delivery.

Acknowledgement

I would like to thank Tommy Cedervall and Thom Leiding for allowing me do my Bachelor's Project with them. They have been provided invaluable help during every step on the way. I would also like to thank Birgitta Frohm for answering numerous questions during lab work. A special thanks goes out to Filip Bäckström and Elvira Oskarsson for giving feedback and company during the writing process.

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Appendix

DLS protocol 1

1. Prepare 7 different IgG solutions (0.005, 0.01, 0.05, 0.1, 0.5, 1 and 3 mg/ml).
2. Prepare 4 different PS-COOH solutions (0.5, 0.1, 0.05 and 0.01 mg/ml).
3. 90 µl of the different IgG concentrations were pipetted according to table 1.
4. 10 µl of the different NP concentrations were pipetted according to table 1.
5. Incubate at 25 C for 20 minutes.
6. Analyze data

Probe Drum Settings

Sample volume: 900 µl
Temperature: 25 C
Baseline: 60 seconds
Concentration titration
Injection of volume. End: 100 µl in xxx µl aliquots.
Stirring speed: 3
Stirring 5 seconds
Spectra during equilibrium
Titrant: PS
From WL 300 to 650
Average 16
Script: [1,395:405]

xxx = 100, 50, 33.33, 20, 5 or 1

DLS protocol 2

1. Prepare 3 different IgG solutions (0.005, 0.01 and 0.05 mg/ml).
2. Prepare 4 different PS-COOH solutions (0.5, 0.1, 0.05, 0.01 mg/ml).
3. 90 µl of the different IgG concentrations were pipetted according to table 2.
4. 10 µl of the different NP concentrations were pipetted according to table 2.
5. Incubate at 25 C for 20 minutes.
6. Analyze data

Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	H2O 0.5 mg/ml NP	H2O 0.5 mg/ml NP	H2O 0.5 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.05 mg/ml NP	H2O 0.05 mg/ml NP	H2O 0.05 mg/ml NP	H2O 0.01 mg/ml NP	H2O 0.01 mg/ml NP	H2O 0.01 mg/ml NP
B	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP	0.005 mg/ml IgG 0.01 mg/ml NP	0.005 mg/ml IgG 0.01 mg/ml NP	0.005 mg/ml IgG 0.01 mg/ml NP
C	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP	0.01 mg/ml IgG 0.01 mg/ml NP	0.01 mg/ml IgG 0.01 mg/ml NP	0.01 mg/ml IgG 0.01 mg/ml NP
D	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP	0.05 mg/ml IgG 0.01 mg/ml NP	0.05 mg/ml IgG 0.01 mg/ml NP	0.05 mg/ml IgG 0.01 mg/ml NP
E	0.1 mg/ml IgG 0.5 mg/ml NP	0.1 mg/ml IgG 0.5 mg/ml NP	0.1 mg/ml IgG 0.5 mg/ml NP	0.1 mg/ml IgG 0.1 mg/ml NP	0.1 mg/ml IgG 0.1 mg/ml NP	0.1 mg/ml IgG 0.1 mg/ml NP	0.1 mg/ml IgG 0.05 mg/ml NP	0.1 mg/ml IgG 0.05 mg/ml NP	0.1 mg/ml IgG 0.05 mg/ml NP	0.1 mg/ml IgG 0.01 mg/ml NP	0.1 mg/ml IgG 0.01 mg/ml NP	0.1 mg/ml IgG 0.01 mg/ml NP
F	0.5 mg/ml IgG 0.5 mg/ml NP	0.5 mg/ml IgG 0.5 mg/ml NP	0.5 mg/ml IgG 0.5 mg/ml NP	0.5 mg/ml IgG 0.1 mg/ml NP	0.5 mg/ml IgG 0.1 mg/ml NP	0.5 mg/ml IgG 0.1 mg/ml NP	0.5 mg/ml IgG 0.05 mg/ml NP	0.5 mg/ml IgG 0.05 mg/ml NP	0.5 mg/ml IgG 0.05 mg/ml NP	0.5 mg/ml IgG 0.01 mg/ml NP	0.5 mg/ml IgG 0.01 mg/ml NP	0.5 mg/ml IgG 0.01 mg/ml NP
G	1,0 mg/ml IgG 0.5 mg/ml NP	1,0 mg/ml IgG 0.5 mg/ml NP	1,0 mg/ml IgG 0.5 mg/ml NP	1,0 mg/ml IgG 0.1 mg/ml NP	1,0 mg/ml IgG 0.1 mg/ml NP	1,0 mg/ml IgG 0.1 mg/ml NP	1,0 mg/ml IgG 0.05 mg/ml NP	1,0 mg/ml IgG 0.05 mg/ml NP	1,0 mg/ml IgG 0.05 mg/ml NP	1,0 mg/ml IgG 0.01 mg/ml NP	1,0 mg/ml IgG 0.01 mg/ml NP	1,0 mg/ml IgG 0.01 mg/ml NP
H	3.0 mg/ml IgG 0.5 mg/ml NP	3.0 mg/ml IgG 0.5 mg/ml NP	3.0 mg/ml IgG 0.5 mg/ml NP	3.0 mg/ml IgG 0.1 mg/ml NP	3.0 mg/ml IgG 0.1 mg/ml NP	3.0 mg/ml IgG 0.1 mg/ml NP	3.0 mg/ml IgG 0.05 mg/ml NP	3.0 mg/ml IgG 0.05 mg/ml NP	3.0 mg/ml IgG 0.05 mg/ml NP	3.0 mg/ml IgG 0.01 mg/ml NP	3.0 mg/ml IgG 0.01 mg/ml NP	3.0 mg/ml IgG 0.01 mg/ml NP

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	H2O 0.5 mg/ml NP	H2O 0.5 mg/ml NP	H2O 0.5 mg/ml NP	H2O 0.5 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.1 mg/ml NP	H2O 0.05 mg/ml NP	H2O 0.05 mg/ml NP	H2O 0.05 mg/ml NP	H2O 0.05 mg/ml NP
B	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.5 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.1 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP	0.005 mg/ml IgG 0.05 mg/ml NP
C	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.5 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.1 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP	0.01 mg/ml IgG 0.05 mg/ml NP
D	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.5 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.1 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP	0.05 mg/ml IgG 0.05 mg/ml NP
E	2 µl x5 0.005 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.005 mg/ml IgG 0.05 mg/ml NP
F	2 µl x5 0.01 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.01 mg/ml IgG 0.05 mg/ml NP
G	2 µl x5 0.05 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.5 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.1 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.05 mg/ml NP	2 µl x5 0.05 mg/ml IgG 0.05 mg/ml NP
H												