

The background of the cover is a high-magnification electron micrograph showing numerous spherical gold nanoparticles. Many of these particles are surrounded by a dense, irregular layer of protein molecules, forming a protein corona. The particles vary in size and are distributed across the field of view, with some appearing as bright, well-defined spheres and others as more diffuse, protein-coated structures. The overall color palette is a range of gold and yellow tones.

The Effect of Protein Corona on Gold Nanoparticles Aggregation

DEPARTMENT OF BIOCHEMISTRY | CMPS | FACULTY OF SCIENCE | LUND UNIVERSITY
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LUND UNIVERSITY

Department of Biochemistry and Structural Biology

**The Effect of Protein Corona
on
Gold Nanoparticles Aggregation**

Master Thesis

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Dedicated to

My parents and brothers

My husband, Mostafa

My lovely son, Belal

Popular Science

Nanotechnology is a rapidly growing field. It includes fabrication of very small particles (nanoparticles). There are different types of nanoparticles depending on the material source. Some nanoparticles are used as a vehicle to deliver drugs to cells (e.g. cancer cells). Due to their large surface area relative to their volume, they can carry large amount of drugs. Nanoparticles can aggregate in salty solutions as in normal human blood. Specific molecules can be added to the particles surface to prevent aggregation. One of the widely used particles is gold nanoparticles. They can have different shapes like spheres and rods. One of the advantages of using gold nanoparticles is that the behavior of the particles in the solution can be detected primarily by the color. A purple colored means that small particles are stable in solution. While grey color is an indication of particle aggregation. When a particle enters blood the particle surface starts to interact with components in blood. Some components interact strongly while other interacts weakly with the particle surface. Proteins is one component in blood that often interacts with nanoparticles. Some proteins aggregate the particles and others not. Different techniques are used to measure the size of the particles in protein rich medium. Comparing the particles size naked particles in protein rich medium with protein conjugated particles, gives information about if the protein cause aggregations of the particles or not. Studying the effect of proteins on nanoparticles is important to evaluate the particle toxicity, safety, and suitability as drug carrier.

Abbreviations

NPs	Nanoparticles
PEG	Polyethylene glycol
GNPs	Gold nanoparticles
H ₂ AuCl ₄	Chloroauric acid
MPS	Mononuclear phagocytic system
PGB1	B1 immunoglobulin binding domain of streptococcal protein G
IgG	Immunoglobulin G
DLS	Dynamic light scattering
FCS	Fetal calf serum
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDB	Protein data bank

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Abstract

Nanoparticles (NPs) are the particles between 1 and 100 nm. They are widely used in the medical field as delivery vehicles, therapeutics and contrast agents in cancer diagnosis and treatment. There are different types of NPs divided according to the source of used materials. The agglomeration of NPs can be prevented by adding different polymers to their surfaces. The optical properties of gold nanoparticles (GNPs) make them a good system for following the behaviour of NPs *in vivo*. The surface properties are determined according to the purpose and type of the target. The protein layers covering GNPs is called corona. The composition of corona depends on the particle material, size and surface properties. In this study, different methods (DLS, disc centrifuge and SDS-PAGE) were used to investigate the effect of different proteins, including PGB1, IgG and plasma proteins (cow and calf) on the aggregation of GNPs and how these aggregations could be prevented. It was found that PGB1 did not aggregate the particles while IgG did and the amount of aggregation depends on IgG concentration. In addition, PBS aggregated the particles while the water stabilized them. Finally, some experiments were done on carboxylated and biotinylated GNPs trying to conjugate to PGB1 and streptavidin respectively, but the results were unclear and more studies are needed.

1. Introduction

1.1 Nanoparticles and drug delivery system

Nanotechnology is a rapidly growing field. It includes fabrication of materials at the nanoscale level with novel physical/chemical-properties and functions ^[1]. One of the most important applications of nanotechnology is nanomedicine, which focus mainly on development of nanoparticles (NPs), sub-100 nm structures to be used as contrast agents, delivery vehicles or therapeutics in diagnosis and treatment of cancer ^[2]. The importance of NPs in the medical field is due to their relatively large surface area, which enable them to bind and carry other compounds like proteins, drugs and probes. Although NPs are defined as having dimensions below 100 nm, sometimes, bigger NPs could be needed to carry sufficient amount of the drug. Moreover, for drug delivery, the drug itself may be fabricated in the nanoscale and acts as drug and carrier at the same time instead of using engineered particles. The sources of the materials used to make engineered NPs could be biological like phospholipids, lipids, lactic acid and dextran or could have chemical properties like polymers, carbon and metals. The interactions between NPs and the cells vary according to the type of NPs.

The main goals for designing drug delivery system are the specificity of drug targeting and delivery, reducing the toxicity but saving therapeutic effects at the same time leading to fast development of new safe drugs. To design a drug delivery system, the following information about the drug should be known and studied, (1) drug integration and release, (2) drug stability, (3) biocompatibility, (4) biodistribution and targeting and (5) efficiency. The aims of using NPs as drug vehicle are to improve delivery to the target cells and reduce the toxicity of the free drug to non-target cells. Coating of NPs using different polymers like polyethylene glycol (PEG) can be used to prevent agglomeration of NPs and keep particles in colloidal suspension ^[3]. Pegylated gold nanoparticles (GNPs) is a perfect system to follow *in vivo* the behavior of NPs. This is due to the particles low toxicity, production in monodisperse batches, that can be modified chemically and to their optical properties ^[4]. Many functional groups can be attached to GNPs such as amino group, carboxyl group and biotin. The Choice of functional groups, depends on the type of the target and the purpose ^[5].

1.2 Historical background of GNPs

The GNPs story started when Lycurgus Cup (4th century Roman glass cage cup) was made from colloidal gold, which changed its color depending on the direction of light. Later, this is become the common staining glass method. In the 17th century, Cassius made a purple colored glass by adding tin to gold solution.

Scientific development of nanotechnology actually started when Michael Faraday prepared the first pure sample of GNPs. In 1857, he reduced gold chloride by phosphorous giving a purple color. Faraday was the first who explained what happened when the light incidents on the glass and changed the color. Gold was dispersed in ruby glass uniformly. When the wavelength of the incident light was smaller than the particles, they appeared in different colors compared to their original colors.

In 1902, Zsigmondy invented ultramicroscope to visualize individual particles. He determined the accurate size of the particles and detected interesting properties depending on the size and motion of NPs. He studied the changes in gold colors caused by adding salts and explained that it might be due to aggregation of particles in different sizes and using protective agents like gelatin could prevent aggregation.

GNPs are classified according to shape, size and physical properties. The first type of produced GNPs was nanospheres. After that, other types like nanorods, nanoshells and nanocages were produced. The diameter of gold nanospheres varied from 2 nm to 100 nm which synthesized by reducing HAuCl_4 (Chloroauric acid) solution by different reducing agents. Citrate is a common used reducing agent. The ratio of citrate and gold controls the size of nanospheres^[6].

1.3 The effect of NPs shape, size and surface chemistry

Once NPs enter a biological fluid, proteins start to compete for binding to NPs forming a layer around the particles called corona which can be hard or soft. The strongly adsorbed NPs proteins represent the hard layer while weakly interacting serum proteins represent soft corona layer. The soft layer is dynamic and can be changed during the NPs life cycle *in vivo*. The corona composition depends on particle material, size and surface properties^[7]. It is suggested that the hard corona proteins cover the NPs surface while soft corona proteins bind to the hard corona by weak protein-protein interactions^[8].

In vitro

NPs can interact with different structures on the cell surface membrane. NPs can bind to receptors on the membrane without entering the cell and induce a signal cascade. NPs can enter through receptor-mediated endocytosis and interact with specific organelles. Another way is that NPs interact with different organelles (nucleus, mitochondria, etc.). They can also bind at unspecific sites on the cell membrane^[9].

There are many factors affecting the behavior of NPs at the nano-bio interface. The nano-bio interface is defined as the dynamic physicochemical interactions, kinetics and thermodynamic exchanges between the biological component surfaces and the nanomaterial surface^[10]. The size and shape of NPs have a direct effect on the cellular uptake. The highest uptake for NPs larger than 100 nm is rods, followed by spheres, cylinders and cubes. In contrast, sub-100 nm NPs spheres have higher uptake than rods. The interactions of non-spherical NPs with the cell are more complicated than spherical particles.

For example, ligand coated rod shaped NPs can interact with the cell by two different orientations, the long axis will interact with more surface receptors than the short axis.

Beside the shape and size of NPs the composition plays an important role in the total cell uptake. For example, the endocytosis rate of single walled carbon nanotubes and GNPs, each 50 nm in diameter, are 10^{-3} and 10^{-6} min^{-1} respectively. This difference can be due to the different properties of carbon compared to gold. In addition, the type of ligand used to coat NPs affect the biological response (i.e. the uptake and cytotoxicity when two different proteins coated NPs targeting the same receptor are different).

In vivo

Over the past 20 years, animal studies on NPs behavior and distribution in the tissue showed that there are some parameters controlling the behavior of NPs. The surface chemistry of NPs determines the type of the protein bound to the surface of NPs and the strength of interactions. It is found that the blood half-life for neutral NPs is higher than positive charged NPs, which cause some problems like platelet aggregation and hemolysis. When NPs administrated into the blood, they are removed by phagocytic cells of the mononuclear phagocytic system (MPS) within minutes or hours. By adding PEG to the NPs, the blood half-life increases. For GNPs, the blood half-life increases by increasing the length of PEG as the protective layer becomes thicker.

Besides PEGylation of NPs, the shape, size and surface chemistry also control the blood half-life. Rod shaped micelles have ten times longer circulation lifetime than spherical micelles. The diameter of intravenously administrated NPs determines their biodistribution and pharmacokinetics according to different sizes of interendothelial pores coating the blood vessels. NPs diameter 6 nm or smaller, are quickly removed from the body due to excretion by kidneys. NPs with 200 nm diameter accumulate in the liver and spleen where they are removed by the MPS cells. NPs can also accumulate in the tumor tissues. NPs diameter controls their overall accumulation and the tumor penetration depth. NPs diameter between 30 and 200 nm is favorable to produce long circulating NPs, accumulating inside tumor tissues ^[9].

Sonavane et al. studied the distribution of different sizes of GNPs (15, 50, 100 and 200nm) in the mice tissues. They found that 15 nm GNPs had higher distribution in the tissues than larger GNPs. They were accumulated in liver, lung, spleen and kidney respectively. 15 and 50 nm GNPs passed the blood brain barrier and accumulated in the brain ^[11].

1.4 The effect of GNPs conjugation on the attached proteins

When the protein binds to NPs, it undergoes a conformational change, which effects on its function by improving or losing it. For example, when trypsin adsorb to silica surface, the enzymatic activity of trypsin decreased 10 times and when it adsorb to polystyrene surface, it lost its proteolytic function completely, which could be due to extensive conformational changes. The trypsin function was regained when the trypsin molecules were removed from

polystyrene and silica surfaces ^[12]. Sometimes functionalized nanoparticles lose its targeting ability when placed in biological environment. For example, transferrin conjugated particles could not bind to the target receptors on the cells as the proteins in the media shielded them completely ^[13].

1.5 B1 Immunoglobulin-Binding Domain of Streptococcal protein G (PGB1)

Many infective bacteria have proteins on their surface that allow them to bind strongly to immunoglobulins and other host proteins. Protein G, a streptococcal protein, binds to the Fc region of human immunoglobulin G (IgG) (appendix II, fig.16). It consists of small binding domains separated by linkers. Near the C terminus there is a cell wall anchor. Two (three in some strains) of the domains bind to IgG while the other two or three domains bind to serum albumin. These binding interactions help the pathogen to escape from the immune response of the host. The advantage of IgG binding of protein G domains is used to purify antibodies. The domains are called as B1, B2, etc. (numbering from N terminus). PGB1 is a small protein 6 kDa with 56 residues, consists of four stranded β sheets and single α helix (appendix II, fig.17) ^[14]. In this project, PGB1 is used to conjugate with GNPs

1.6 The interactions between colloidal GNPs and human plasma proteins

Dobrovolskaia et al. examined the size and charge of 30 and 50 nm colloidal GNPs after 30 minutes incubation with human blood plasma by Dynamic Light Scattering (DLS) ^[15]. They found that the hydrodynamic diameter for 30 and 50 nm GNPs increased by approximately 50 nm after the incubation. When the particles bound plasma proteins were digested by trypsin, the particles size returned to approximately their pre-incubation diameter. GNPs surface charge was measured also before and after plasma incubation. The mean particle surface charge increased after incubation (becomes less negative) for both particles sizes.

1.7 What is driving the aggregation of NPs in the blood?

Cukalevski et al. studied the effect of IgG and fibrinogen on the aggregation of NPs ^[16]. Sulfonated polystyrene NPs were incubated with cow serum, depleted IgG cow serum and fetal calf serum (FCS). The absorbance was measured at 400 nm. Most NPs aggregation occurred in the cow serum followed by depleted IgG cow serum and FCS. When isolated bovine IgG was added to the depleted IgG cow serum and FCS the aggregation increased which means that IgG is playing an important role in NPs aggregations. The particles also incubated with fibrinogen. It was found that fibrinogen also caused an aggregation of NPs and by adding fibrinogen to FCS, the aggregation increased. The difference between IgG and fibrinogen is that fibrinogen undergoes structural changes when it binds to the NPs unlike IgG that does not have large structural changes after binding to NPs

1.8 Theory behind used methods

1.8.1 Dynamic Light Scattering (DLS)

DLS is a physical technique used to measure the hydrodynamic size (radius or diameter), including any hydration of solvent layer surrounding the particles, by illuminating the particles with laser light. The particles are moving randomly in the solution all the time (Brownian motion) depending on the particle size (the bigger size, the slower movement), solvent viscosity (the more viscosity, the slower particles movements) and measuring temperature (the higher temperature, the faster particles movements). By fixing temperature and viscosity, the size of the particles can be measured according to the variations of the scattered light ^[17].

1.8.2 Disc centrifuge

The sedimentation of the particles is used to characterize the distribution of the particle size. Stoke's law is used to detect the size distribution of spherical particles by measuring the required time for the particles to set at a known distance in a known viscosity and density fluid. The centrifugal sedimentation increases the range of sedimentation studies including very small particles.

The following equation is the Stoke's law for sedimentation,

$$D = \left\{ \frac{18\eta \ln(R_f/R_o)}{(\rho_p - \rho_f) \omega^2 t} \right\}^{0.5}$$

where, D is the particle diameter(cm) , η is the fluid viscosity (poise), R_f and R_o are final and initial radius of rotation respectively (cm), ρ_p and ρ_f are the density of the particle and fluid respectively (g/ml), ω is the rotational velocity (radians/sec) and t is the required time for sedimentation from R_o to R_f (sec).

When the centrifuge run at constant speed and temperature, all parameters in the equation are constant except the time. The arrival time to the detector depends on the size and density of spherical particles such as gold surrounded by protein shells. The coated protein particles sediment slower than uncoated protein particles while the aggregated particles will sediment more rapidly ^[18]. Disc centrifuge can separate particles with less than 5% size difference. The sedimentation is stabilized by a slight water density gradient which are often two different sucrose concentrations determined according to the particles density ^[19].

1.8.3 One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a common technique used to separate biological macromolecules (proteins and nucleic acids) in a polyacrylamide gel according to the size. An electric field is applied on the polyacrylamide gel, which causes migration of SDS-treated proteins towards the anode. The proteins are treated with SDS in order to have a uniform rod shape and to have a constant negative charge to size ratio, so the proteins move through the gel according to their size only (i.e. larger proteins move slower than small proteins). The size of the protein could be detected by using SDS-treated calibration proteins. After the electrophoresis process, the proteins can be detected by staining with Coomassie Brilliant Blue ^[20].

1.9 Aim of the work

- Study the different effects of water and phosphate buffered saline (PBS) on GNPs aggregations and how these aggregations can be prevented.
- Conjugation of GNPs with PGB1.
- Study plasma proteins (cow and calf) influence on PGB1 conjugated GNPs.
- Investigate the effect of IgG on the aggregation of GNPs.

- Study the effect of surface GNPs on conjugation process (citrate stabilized, carboxylated and biotinylated GNPs).
- Compare two different methods used to measure the particles size (DLS and disc centrifuge).

2. Materials and Methods

2.1 Used materials

Citrate stabilized GNPs with 24 and 26 nm were synthesized with concentrations 1.13 and 25 mM respectively ^[21]. In all the experiments, they were diluted 10 times. Cow and FCS were purchased from Innovative Research. PGB1 was prepared as described in [22]. Human IgG was purchased from Lee Biosolutions with >98 % purity. Carboxylated and biotinylated, 20 nm GNPs supplied in USP grade purified water were purchased from Cytodiagnosics. Streptavidin with 0.5mg/ml stock concentration was used. Alexa flour 488 was purchased from life Technologies. All the dilutions were done using 1xPBS and in some experiments, water was used.

2.2 Used Methods

DLS

Used DLS instrument was Wyatt Dyna pro plate reader II connected to Dynamics software for analyzing DLS measurements. Corning® 96 Well Polystyrene plate was used. The measuring temperature was 25°C. The volume of the measured samples was 100 µl.

Disc centrifuge

CPS disc centrifuge model DC24000 was used to measure the particles in range 0.01µm to 0.3µm. The gold density is 19.3 g/ml with refractive index 0.47 and absorption 1. According to gold density, the appropriate aqueous sucrose density gradient is 8 and 24 % with 14 ml total volume (average sucrose density = 1.064 g/ml). After injecting gradient in the disc, 0.5ml dodecane was injected to prevent evaporation of the fluid from the rotating disc. According to the particles size and density, the recommended speed was 15396 RPM with 10 minutes running. Instrument calibrated by 0.483µm PVC with density 1.385g/ml. The volume of the measured samples was 100 µl ^[23].

SDS-PAGE

12% acrylamide gel was used. The samples were centrifuged for 10 minutes at 13000 rpm, then the supernatants were discarded and the pellet washed 2 times with 400µl PBS and

transferred to new Eppendorf tubes, then the pellet was incubated with 10 μ l SDS for 5 minutes at 85°C. 10 μ l was loaded in each well of the gel.

2.3 Experiments

Citrate-stabilized GNPs

The size of 24 nm citrate stabilized GNPs was measured in water and PBS by DLS. The particles size in PBS before and after adding 0.1 mg/ml PGB1 was measured by disc centrifuge and DLS. To study the effect of cow serum on the aggregation of GNPs conjugated to PGB1, different concentrations of cow serum (1, 5, 10, 30 and 50%) was added to GNPs incubated 30 minutes with 1mg/ml PGB1. Then, the samples was incubated another 30 minutes. The control samples were GNPs dispersed in PBS, 1 mg/ml PGB1 with GNPs, 50% cow serum and 50% cow serum with 1 mg/ml PGB1. The size was measured by disc centrifuge. The proteins bound to conjugated GNPs were examined and separated by SDS-PAGE. The negative and positive controls were 30% cow serum and 30% cow serum with GNPs respectively. Another experiment was done using water as a buffer and 0.1 mg/ml PGB1 with GNPs, which incubated 30 minutes then 5, 10, 60 and 80% cow serum, were added. The size was measured by DLS with GNPs and 0.1 mg/ml PGB1 conjugated GNPs as controls. SDS-PAGE was run for 1mg/ml PGB1 with 0.113 mg/ml GNPs and 10, 60 and 80% cow serum. The controls were 0.1 mg/ml PGB1 with 80% cow serum (negative control) and GNP with 80% cow serum (positive control). This experiment was repeated replacing cow serum by FCS. To investigate the interactions between GNPs and cow serum and FCS, different concentrations of cow serum (1, 5, 10, 50 and 80%) were prepared using PBS, then GNPs were added and incubated for 60 minutes. The total volume of the sample was 200 μ l, divided equally for disc centrifuge and DLS measurements. The same experiments had been done replacing cow serum by FCS. The effect of IgG on the aggregation of GNPs was studied by preparing different concentrations of IgG (0.001, 0.0.1, 0.1, 0.5 and 1 mg/ml) and measuring the size by DLS. Different PGB1 concentrations (0.1, 0.5, 1, 2 and 3 mg/ml) with GNPs in water and PBS were incubated 30 minutes. The total volume of the sample was 200 μ l, divided equally between disc centrifuge and DLS to measure the particles size. The size of GNPs in water and PBS was measured after one year from DLS measurement. This time the size was also measured by disc centrifuge.

Another GNPs with concentration 2.5 mM and 26 nm were used to check the reason of the aggregation, is it due to IgG or the particles itself were aggregated. Most of the experiments were repeated using these particles including the particles size, PGB1 conjugated particles and PGB1 conjugated particles in cow serum.

Carboxylated GNPs

PGB1 was conjugated to 20 nm carboxylated particles according to cytodiagnostics protocol ^[24]. Then, different concentrations of cow serum (10, 20, 40, 60 and 80%) were incubated 30 minutes with the conjugated and unconjugated carboxylated GNPs. Size was measured by DLS and SDS-PAGE was run for these samples.

Biotylated GNPs

Biotinylated GNPs, 20 nm were incubated 30 minutes with different streptavidin to biotin ratios (0.1, 0.5, 1, 1.5, 2 and 5), measuring the size by DLS. Different concentrations of cow serum (1, 5, 10, 50 and 80%) were incubated for 1 hour with biotinylated GNPs conjugated to streptavidin. The size of the particles was measured by DLS and disc centrifuge. To study the behavior of streptavidin, Alexa flour 488 dye was used to label streptavidin ^[25]. The fluorescence was measured by fluorometer.

3. Results and discussion

Citrate-stabilized GNPs

3.1 Particles size (in water and PBS)

The stability of GNPs in water and PBS was measured by DLS. Fig.1 shows a big difference in the size of the particles dispersed in water and in PBS. The size of GNPs in water was around 28 nm with a small peak at 5560nm, which could be aggregated particles. GNPs in PBS were aggregated giving a monodisperse peak at 1106 nm. NaCl in PBS played an important role in the aggregation of the particles as Na⁺ bound to the carboxylic acid of the citric acid groups at GNPs surface, which neutralized the stabilizing electrostatic forces (E_{ES}) causing van der Waals forces (E_{vdw}) to drive instantaneous and irreversible aggregation of the GNPs, giving a dark grey solution (fig.1) ^[26].

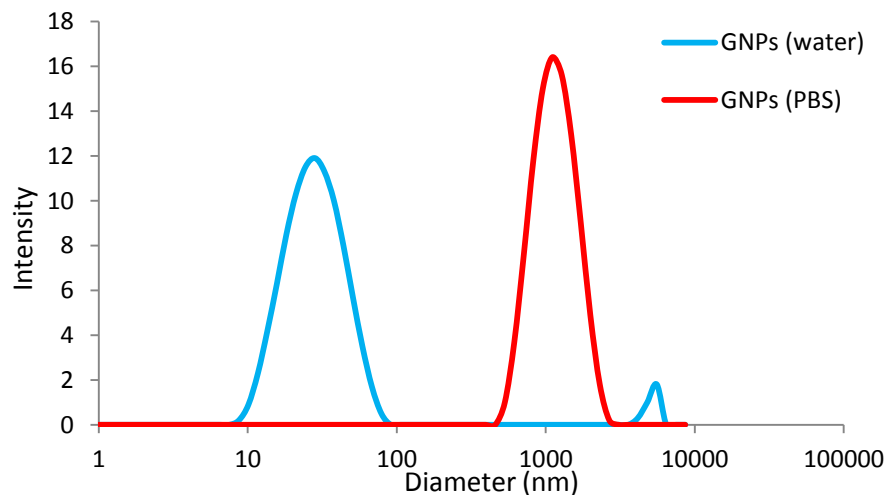


Fig.1: DLS measurements of GNPs in water (cyan) and PBS (red).

3.2 How the aggregation of GNPs could be prevented?

The order of preparing the sample controlled the aggregation of the particles. In fig.2, there was a difference in the color of GNPs between A, B and C. In figure 2A, GNPs were stabilized in the water and did not aggregate (purple color). In figure 2B, GNPs were

aggregated immediately by adding PBS, so the color changed to dark grey. In figure 2C, PBS was added to the particles after adding 1mg/ml PGB1. The aggregation of GNPs in PBS when PGB1 added first and when added after PBS was completely different (fig.3). Scheme 1 suggests what is happening when PGB1 is added first and when it is added after PBS. Once, PBS added to the particles, they started to aggregate (fig.1) but the surface of the particles complex, which exposed to the PBS, could bind to PGB1 protein and cover the outer surface of the complexes. When PGB1 was added first, the particles did not aggregate as PGB1 was forming a layer around the particles and preventing their aggregation so the color of the particles remained purple (fig.2C) ^[26].

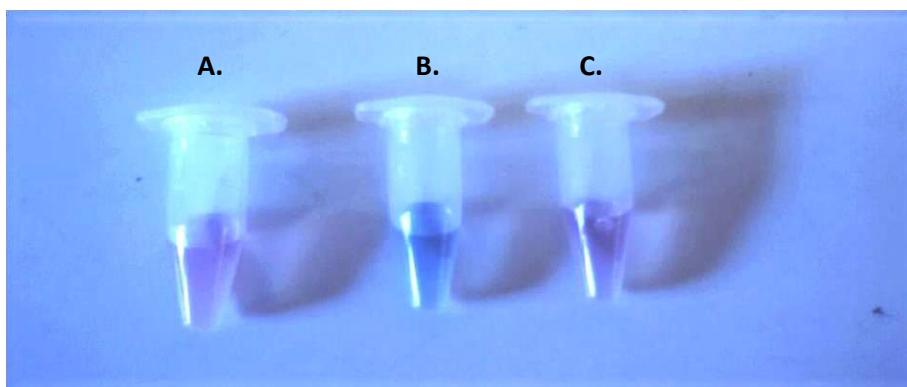


Fig.2:A. GNPs in water ,**B.** GNPs in PBS and **C.** GNPs with 1mg/ml PGB1 and after that PBS were added.

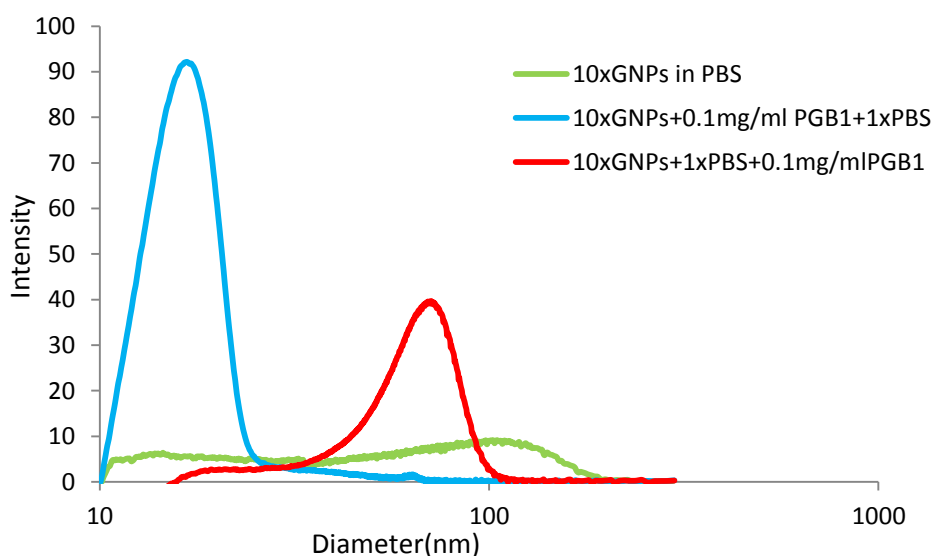
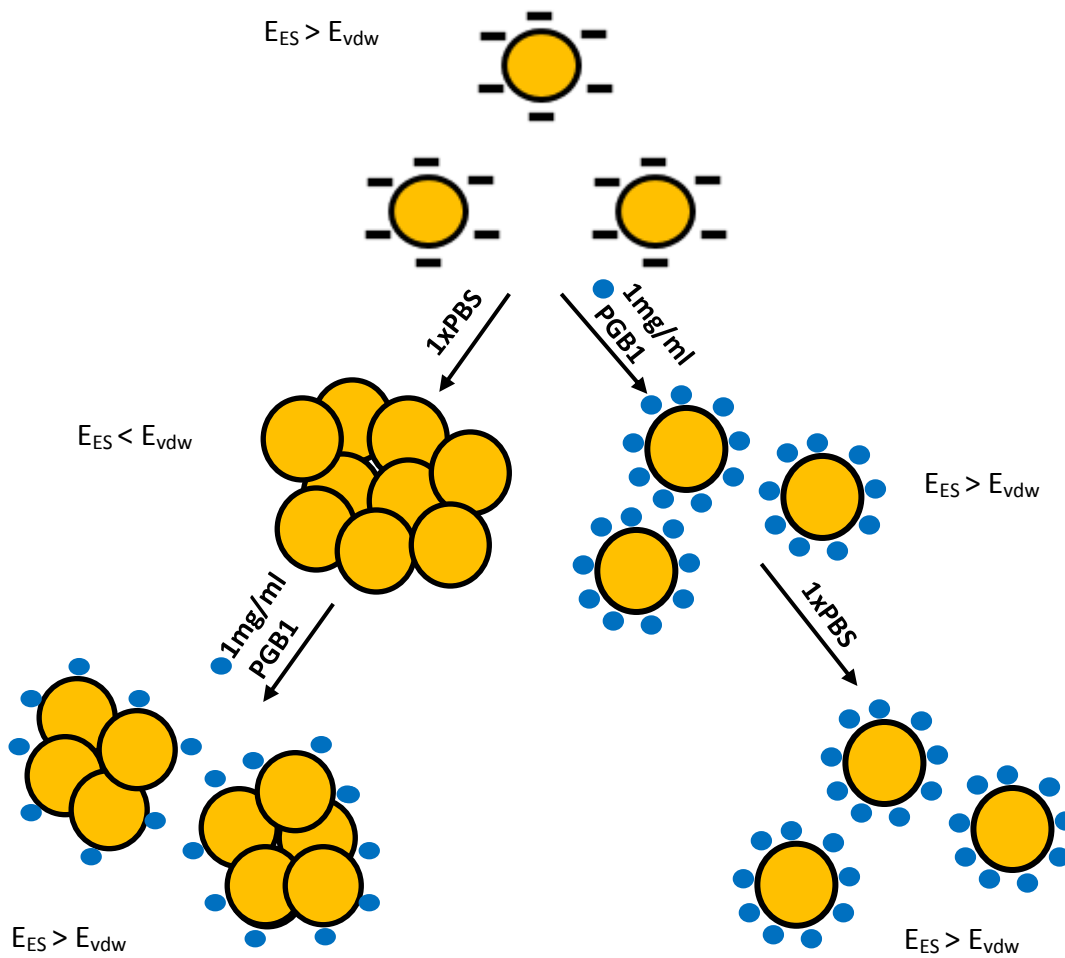


Fig.3: Disc centrifuge measurements of GNPs in PBS (green),GNPs in PBS after adding 0.1mg/ml PGB1(cyan) and GNPs dissolved in PBS and after that 0.1mg/ml PGB1 was added (red).



Scheme1: The aggregation of GNPs in PBS before and after adding 1mg/ml PGB1.

3.3 Effect of cow serum on the aggregation of GNPs conjugated to PGB1

The aggregation of GNPs conjugated to 1mg/ml PGB1 and different cow serum concentrations (1, 5, 10, 30 and 50%) in PBS was measured using disc centrifuge (fig.4). In this experiment, there were four controls, one was only GNPs dissolved in PBS, the second was GNPs with 1mg/ml PGB1, the third was only 50% cow serum and the last one was 50% cow serum with 1mg/ml PGB1. By comparing the first two controls, a big difference was observed as GNPs with PGB1 had a single peak around 20 nm while GNPs sample had a wide peak covering a large range of sizes but with lower intensity, which an indication about the interactions between PGB1 and GNPs. In the last two controls, there was a difference between 50% cow serum sample and 50% cow serum with 1mg/ml PGB1. At 50%, cow serum with 1mg/ml PGB1, there was peak about 70 nm, which an indication about the interaction between PGB1 and IgG in cow serum. At 1% cow serum with PGB1 and GNPs, a very small peak appeared at 15 nm. By increasing cow serum concentration this peak became small and a bigger one appeared around 70 nm until 50 % cow serum at which the

small peak disappeared and only peak around 70 nm appeared, which was exactly the same as 50 % cow serum with PGB1 control sample.

At 1% cow serum almost all PGB1 covered GNPs and at the same time PGB1 interact with serum and that is why the peak was shifted to a smaller size as the protein coated particles moved slower than the uncoated ones [18]. By increasing cow serum, concentration (5, 10 and 30%) two peaks appeared, as part of PGB1 interacts with GNPs and another part with serum so both of the peaks could be seen. Going up to 50% cow serum the small peak disappeared and the curve fit exactly the 50 % cow serum with PGB1 control sample, which means that, may be all PGB1 left the particles and bound to Fc fragment of IgG in cow serum. A negative peak appears in most of the samples, which we cannot explain.

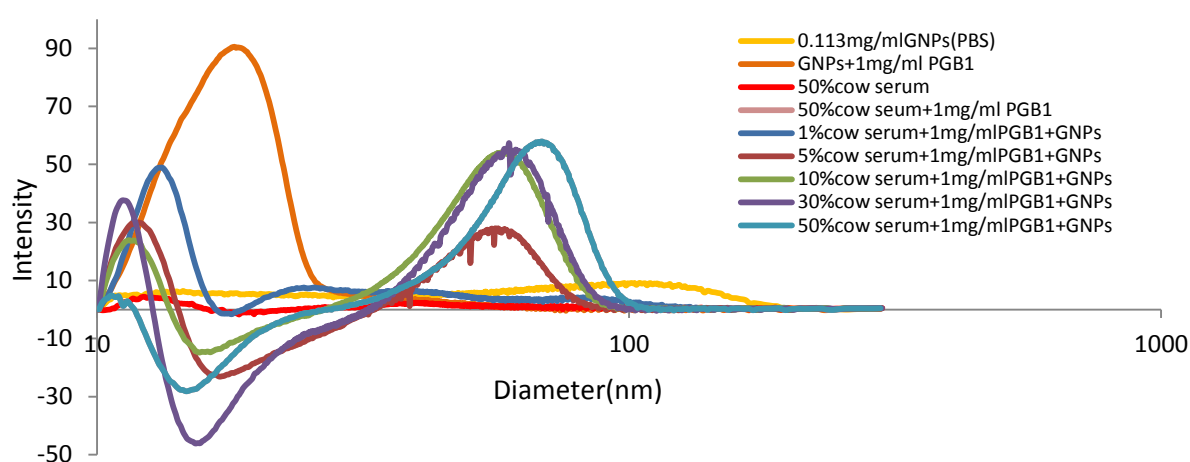


Fig.4: Disc centrifuge measurements of 0.113 mg/mlGNPs with 1mg/ml PGB1and different cow serum concentrations (1, 5, 10, 30 and 50%).

To interpret more clearly the disc centrifuge data and to investigate what was bound to the particles, the particles were centrifuged down and SDS-Page gel was run on the pellet (fig.5). The negative controls were 30% cow serum and 30%cow serum with 1mg/ml. The positive control was 30% cow serum with GNPs.

The first negative control (30% cow serum) had no bands, as nothing was precipitated during centrifugation of the sample. Second negative control (30% cow serum with 1mg/ml PGB1) had two bands, one was around 50 kDa and the other was around 15 kDa. It was thought that, these two bands were for the heavy and light chain of IgG as PGB1 bound to Fc fragment of IgG (this was agree with disc centrifuge curve, fig.4). At 1%, serum there was no bands appeared as the cow concentration was very low and GNPs with PGB1 could not be seen in SDS-PAGE gel (look at appendix I, fig.15). At 5%, there were two bands one for the heavy chain and one for the light chain of IgG, which an indication that there are interactions between GNPs and IgG. At 10%, another band started to appear (albumin) but very faint and the thickness of the two bands of IgG increases as IgG concentration increased. At 30% cow serum, albumin band appeared clearly, while IgG bands were the same. At the highest concentration (50%) IgG bands became faint and albumin was the same.

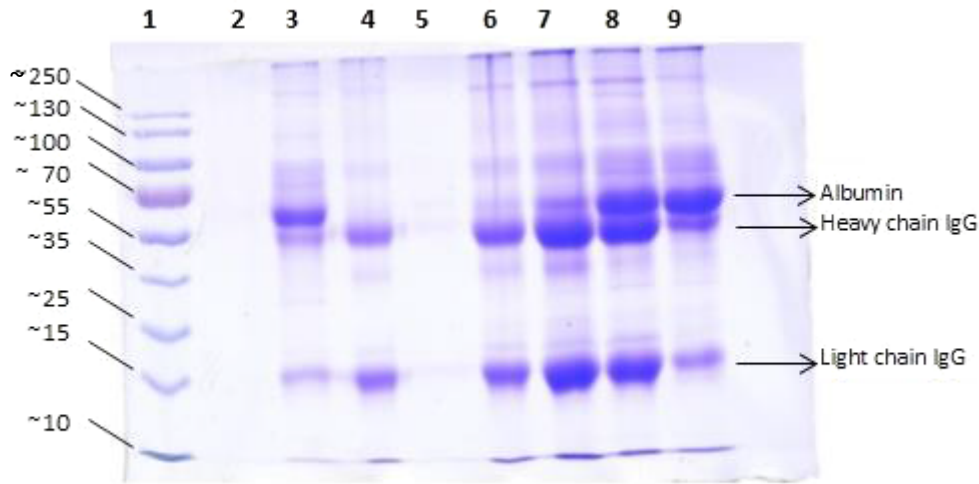
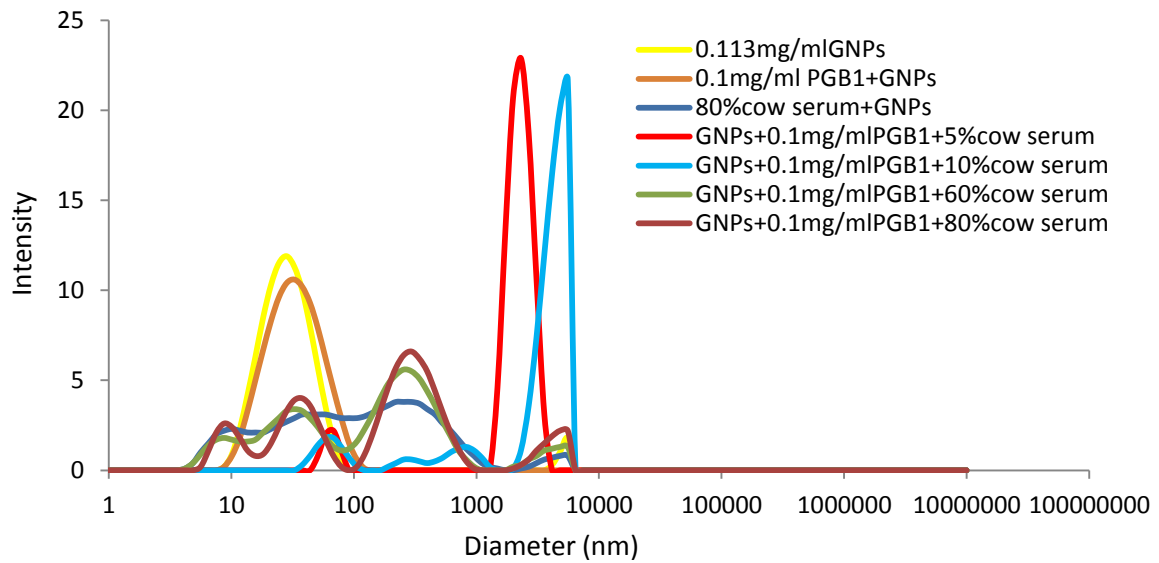


Fig.5: SDS-PAGE gel of GNPs with 1mg/ml PGB1 and different cow serum concentrations. **1.** Ladder, **2.** 30% serum **3.** 30% serum + GNPs, **4.** 30% serum + 1mg/ml PGB1, **5.** GNPs + 1mg/ml PGB1 + 1% serum, **6.** GNPs + 1mg/ml PGB1 + 5% serum **7.** GNPs + 1mg/ml PGB1 + 10% serum **8.** GNPs + 1mg/ml PGB1 + 30% serum. **9.** GNPs + 1mg/ml PGB1 + 50% serum.

From disc centrifuge measurements and SDS-PAGE, it was clear that PGB1 interacted with GNPs and at the same time with cow serum and that is why there were two peaks for all the samples of GNPs with PGB1 and cow serum. Nevertheless, it does not mean that there was no interaction between GNPs and cow serum. It was clear from the gel that by increasing cow serum concentration more IgG bound to the particles and PGB1 until at 50% serum concentration which IgG bands decreased which might mean that PGB1 left GNPs and bound to IgG. As in the disc centrifuge the curve of the control sample (50% cow serum with 1mg/ml) and 1mg/ml PGB1 with 10x GNP and 50% cow serum were the same. Albumin band became more intense as GNPs were stabilized and bound to albumin.

To study, what was happened in the system, the same experiment was done but using lower PGB1 concentration (0.1mg/ml) and replacing PBS by water (fig.6A). At 5% and 10% a large peak appeared at 2305 and 5560 nm respectively. By increasing cow serum concentration the aggregation decreased. Comparing 80% cow serum with GNPs and 80% cow serum with GNPs conjugated to PGB1, it was found that the first sample covering wide range of sizes. While for the sample with PGB1, it covered almost the same range but some Aggregation sizes disappeared in-between as PGB1 covered part of GNPs surface so the surface area of GNPs exposed to cow serum decreased.



B.

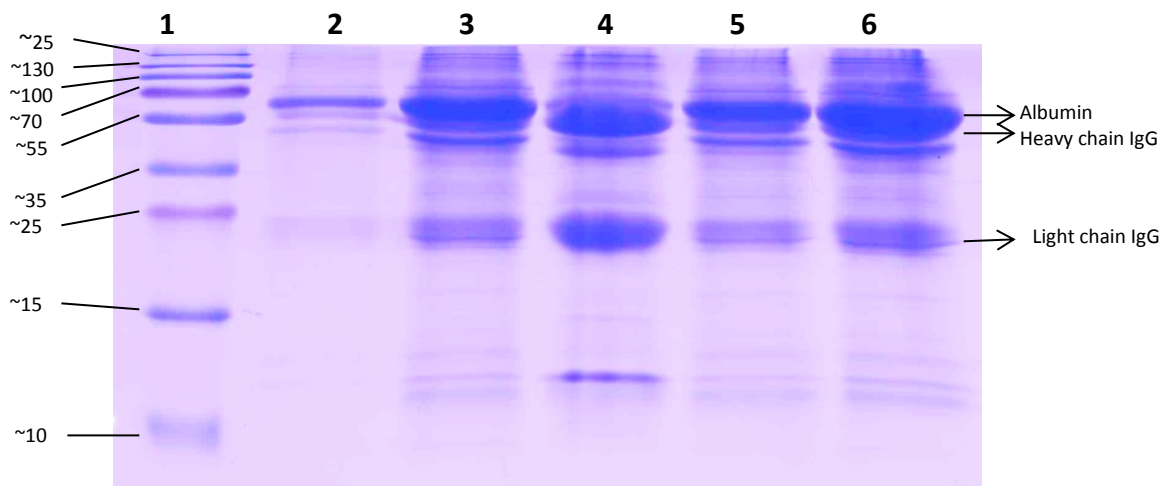


Fig.6: (A) DLS measurements of different cow serum concentrations (5, 10, 60 and 80%) with 0.1mg/ml PGB1 and 10 xGNPs. **(B)** SDS-PAGE gel of different serum concentrations with 0.1mg/ml PGB1 and 0.113 mg/mlGNPs. **1.**Ladder,**2.**0.1mg/mlPGB1+80%cow serum,**3.**80%cow serum+GNPs,**4.**GNPs+0.1mg/mlPGB1+10%cow serum,**5.**GNPs+0.1mg/mlPGB1+60%cow serum,**6.** GNP+0.1mg/mlPGB1+80%cow serum.

SDS-Page was run for 10, 60 and 80% cow serum with GNP conjugated to 0.1mg/mlPGB1 with negative control, 0.1mg/ml PGB1 with 80% cow serum and positive control, GNP with 80% cow serum (fig.6B). At 10%, cow serum the band of heavy and light chain IgG was more intense than 80% and it might be because more IgG bound and aggregated the particles and this was agree with the DLS measurements (fig.6A).

The aggregation of GNP conjugated PGB1 with FCS was lower than with cow serum (fig.7).

Most aggregation was at 80% FCS sample. By decreasing FCS concentration the aggregation decreased but not so much and the smallest aggregation was at 5% FCS (fig.7A), unlike cow serum at which the highest aggregation was at 5 and 10 % cow serum (fig.6A).

SDS-PAGE (fig.7B) of different FCS concentration with GNPs conjugated to PGB1 was totally different from SDS-PAGE of cow serum (fig.6B). Heavy and light chain IgG bands were unclear which an indication of the low amount of IgG bound to GNPs and this was agreed with the DLS measurements (fig.7A).

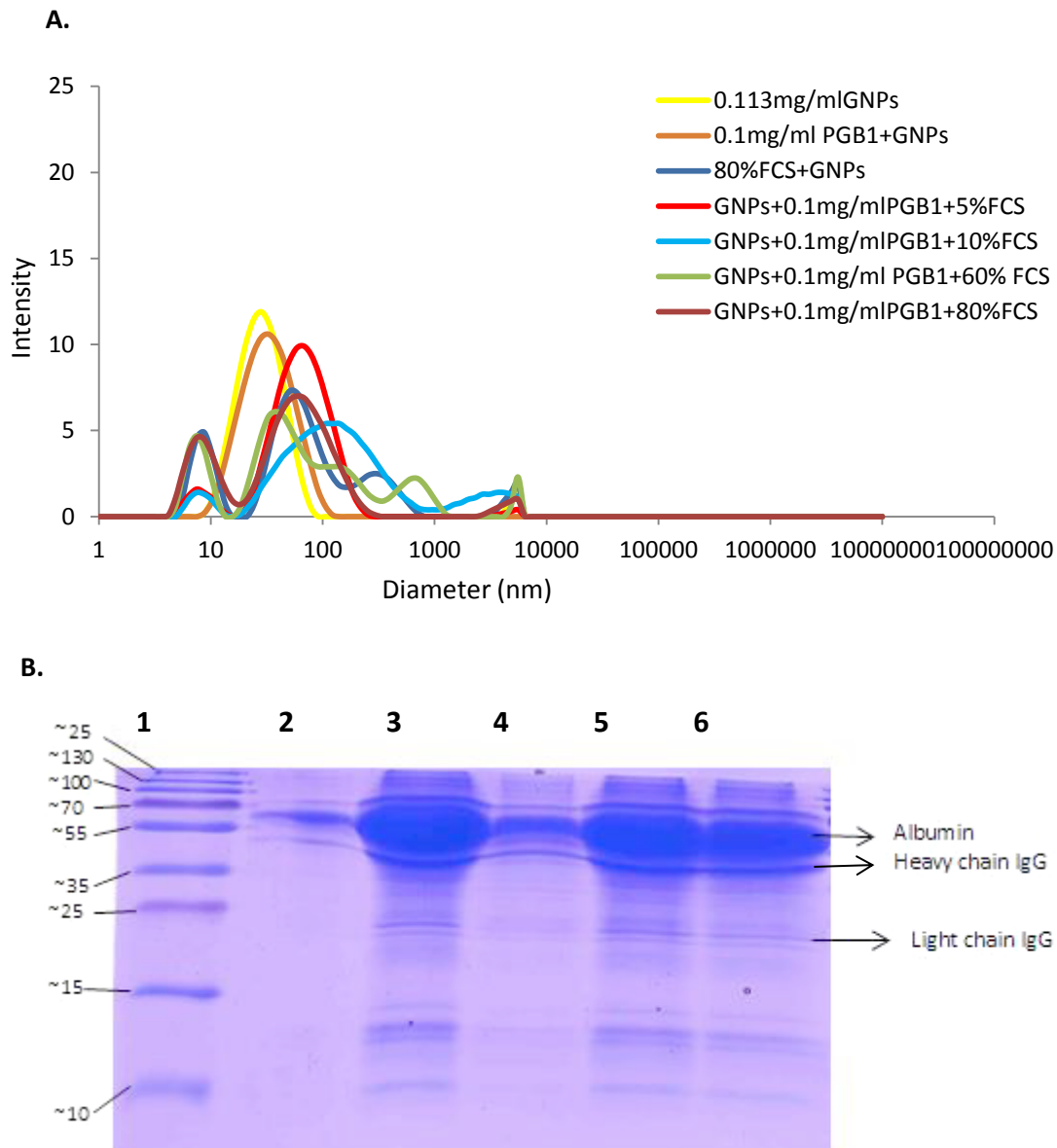


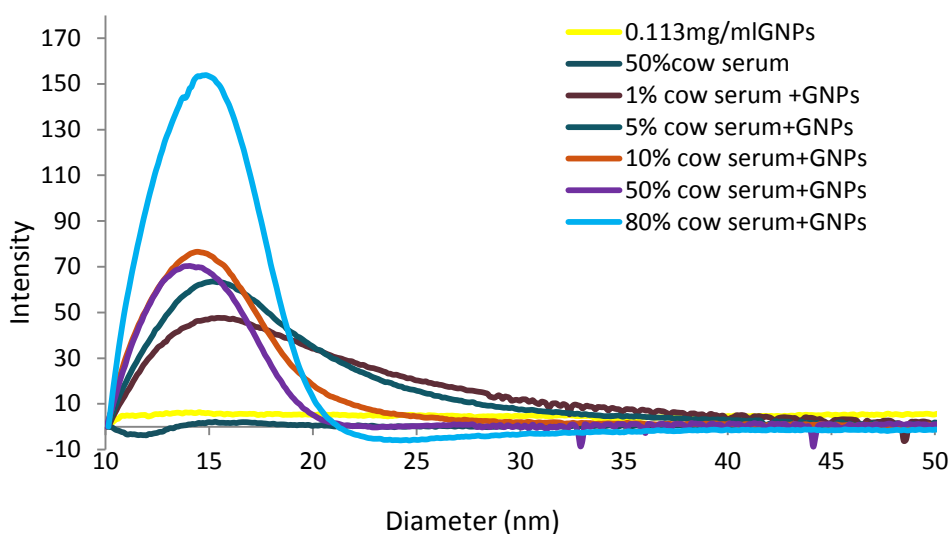
Fig.7: (A) DLS measurements of different FCS concentrations (5, 10, 60 and 80%) with 0.1mg/ml PGB1 and 10 xGNPs. **(B)** SDS-PAGE gel of different FCS concentrations with 0.1mg/ml PGB1 and 0.113 mg/ml GNPs. **1.**Ladder,**2.**0.1mg/mlPGB1+80%FCS,**3.**80%FCS+GNPs,**4.** GNPs+0.1mg/mlPGB1+10%FCS,**5.** GNPs+0.1mg/mlPGB1+60%FCS,**6.**GNPs+0.1mg/mlPGB1+80%FCS.

3.4 The interactions between GNPs and cow serum

In this part, different cow serum concentrations were incubated for one hour with GNPs in PBS. There was a difference between measurements of disc centrifuge and DLS (fig.8). In

disc centrifuge (fig.8A), it seems that there was no aggregation of the particles but there was still interaction between GNPs and cow serum as the intensity increases by increasing cow serum concentration. The aggregation is very clear in DLS measurements (fig.8B). By increasing cow concentration, the size of the particles increased and the peak became flat covering a wide range of different size (at 50% and 80% cow serum). At 50% cow serum couldn't be measured alone in DLS.

A.



B.

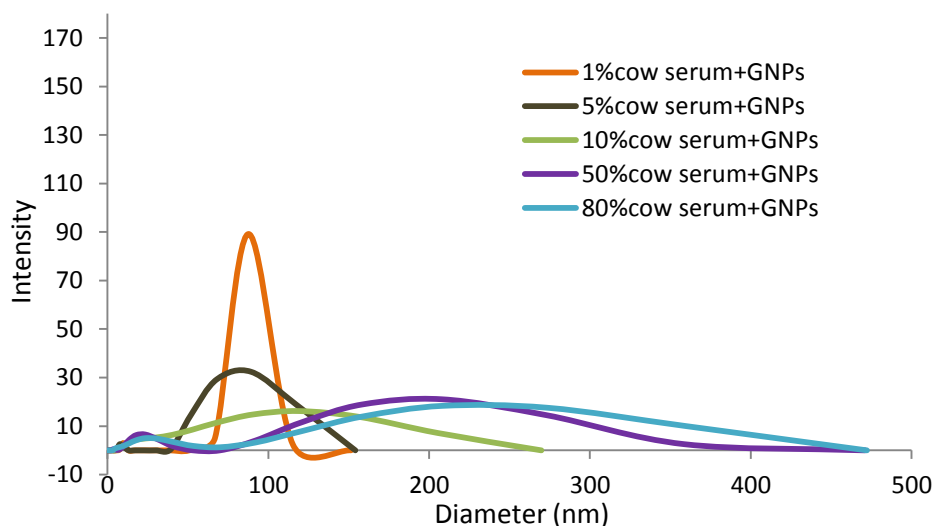


Fig.8: (A) Disc centrifuge and **(B)** DLS measurements of different cow serum (1, 5, 10, 50 and 80%) concentrations with 0.113 mg/ml GNPs in PBS.

3.5 The interactions between FCS and GNPs

Different FCS concentrations were incubated for one hour with GNPs in PBS. At 1% FCS (fig.9A), the peak was broad as the concentration was very low so GNPs was still exposed to PBS which enhance the aggregation. By increasing FCS concentration the peak became sharper as the particles completely surrounded by FCS and bound to the particles. In the DLS the data were completely different as the aggregation decreased by increasing FCS concentration from 1% to 10% but after that at 50 and 80 % the size increased again but not like 1%, still 1% had the biggest size (fig.9B).

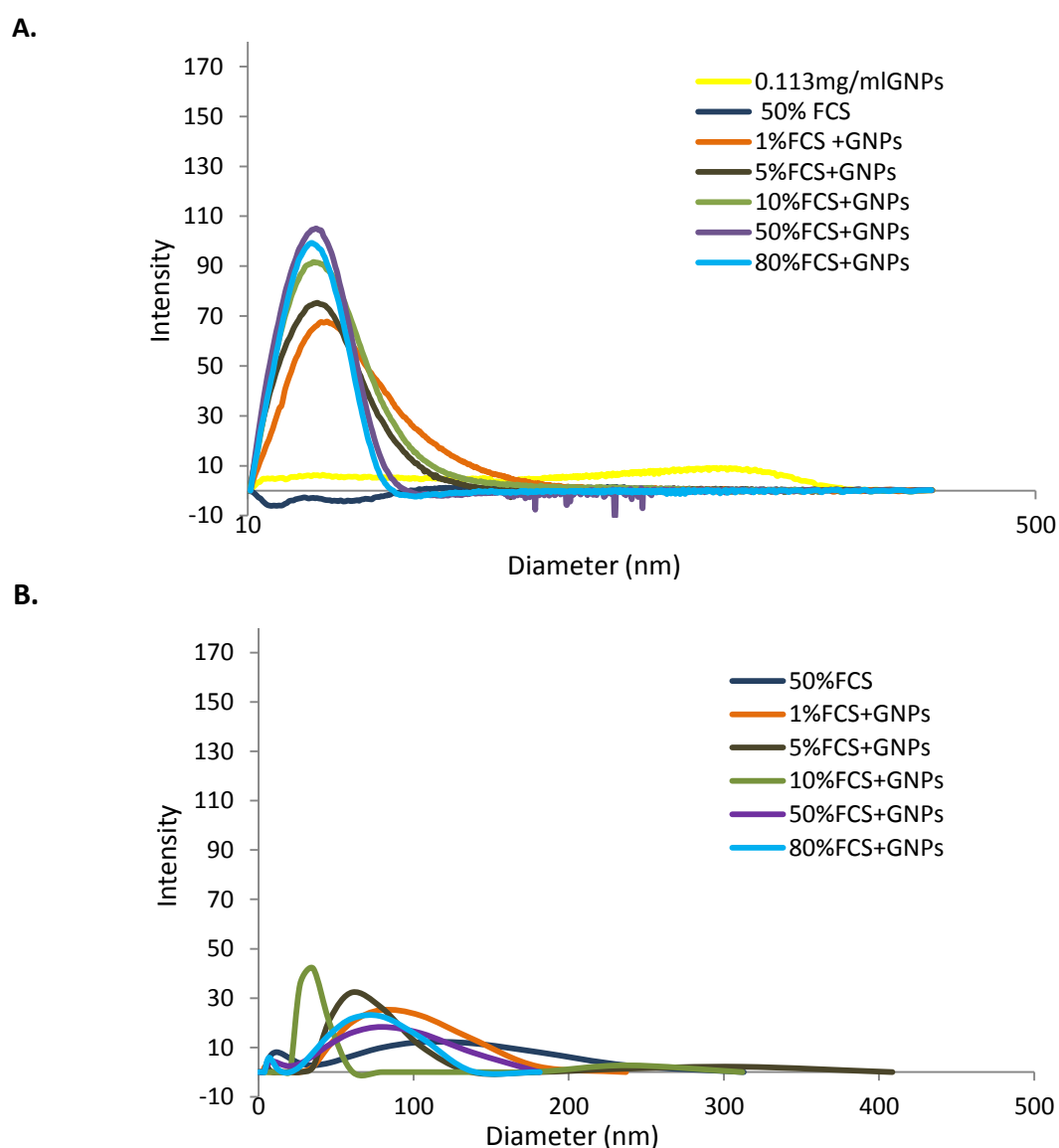


Fig.9: (A) Disc centrifuge and **(B)** DLS measurements of different FCS (1, 5, 10, 50 and 80%) with 0.113 mg/ml GNPs in PBS.

In general, the behaviour of GNPs in cow and calf serum in the DLS measurements (because in disc centrifuge, the size was almost the same) was different as cow serum caused bigger aggregation of the particles than FCS and it might be due to high IgG content in cow serum than calf serum. Therefore, in the next section the effect of different concentrations of IgG on GNPs was studied using water as a buffer to elucidate just the effect of IgG without any aggregation enhancing molecules.

3.6 The Effect of IgG concentration on GNPs aggregations

To study the effect of IgG on GNPs aggregation, GNPs were incubated with different IgG concentrations. The highest aggregation of GNPs was at 0.001mg/ml IgG and by increasing IgG concentration the aggregation of the particles decreased (fig.10).

At low IgG concentrations (0.001 and 0.1 mg/ml), one IgG molecule might bound to more than one GNP at the same time forming a bridge between them causing high aggregation of the particles. By increasing IgG concentration, each IgG molecule bound to GNP, which decreased the probability of IgG to bind to other GNPs, so the aggregation decreased. At very high IgG concentration, there were a high amount of IgG which forming a monolayer around the particles quickly which prevents the aggregation of the particles^[16].

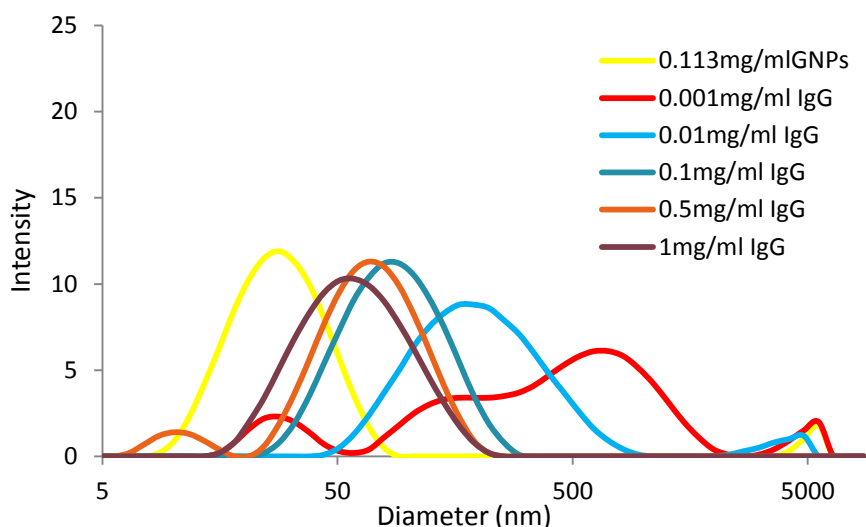


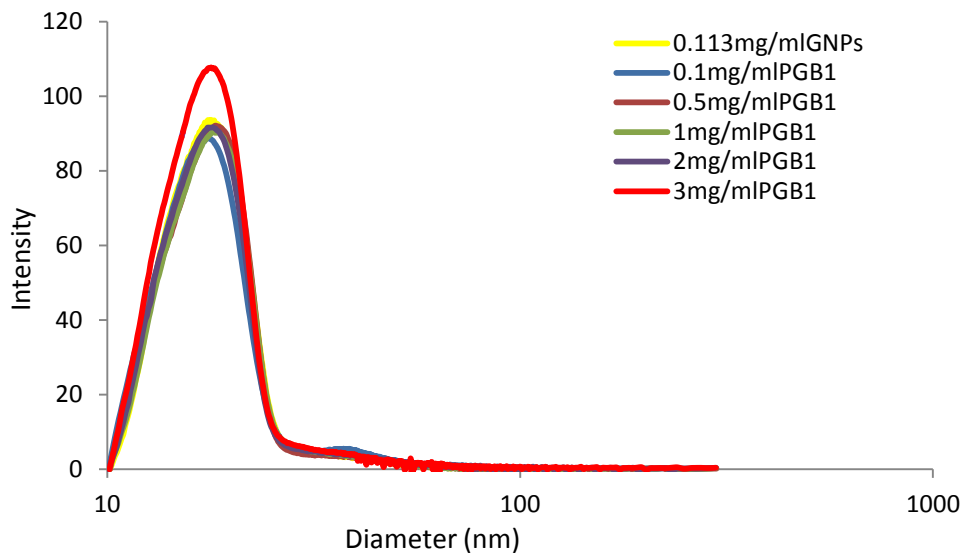
Fig.10: DLS measurements of different IgG concentrations with 0.113 mg/ml GNPs in water.

3.7 The interactions between PGB1 and GNPs

The interaction between GNPs dispersed in water and PGB1 was measured using DLS and disc centrifuge (fig.11). In the disc centrifuge, a small increase in the size by increasing PGB1 concentration was observed which an indication that PGB1 bound to GNPs. This small increase because PGB1 is a small protein. For DLS measurements, the size of the particles increased by increasing PGB1 concentration which also an indication about the interaction

between PGB1 and GNPs. There were differences between results of disc centrifuge and DLS. The size of GNPs could not be measured by DLS, as data were unacceptable while in disc centrifuge it was measured with size around 18 nm. The size of GNPs could be measured by DLS one year before but may be during this time the aggregation increased as in fig.1 there was an aggregation started to grow at 5560 nm.

A.



B.

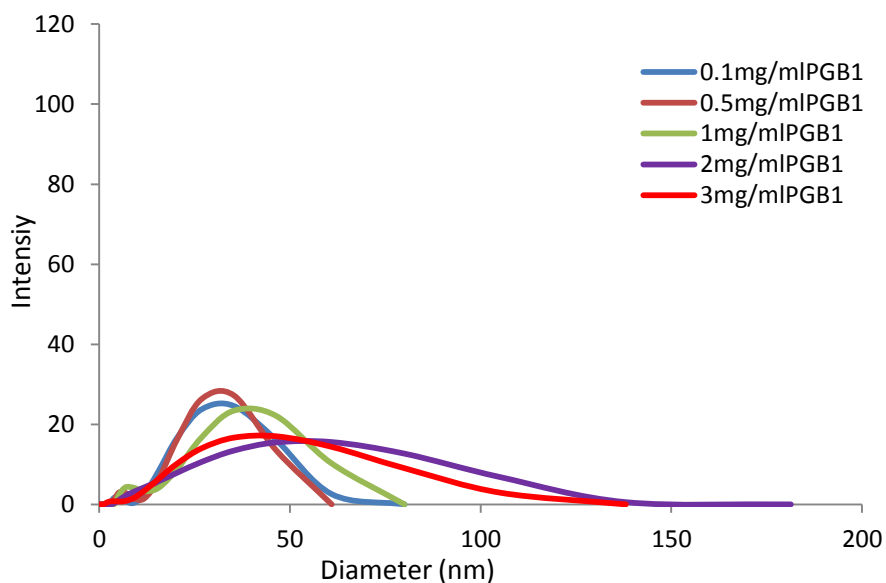
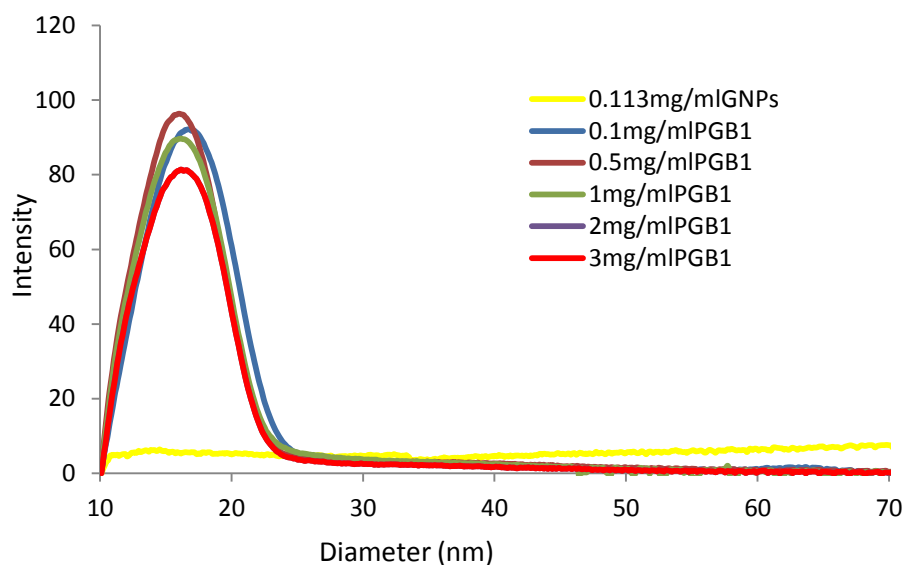


Fig.11: (A) Disc centrifuge and **(B)** DLS measurements of different PGB1 concentrations (0.1, 0.5, 1, 2, and 3mg/ml) with 0.113 mg/ml GNPs in water.

Adding PBS instead of water after adding PGB1 to the particles gave also, a small shift in the peaks in disc centrifuge measurements (fig.12A) but the particles did not aggregate which means that PGB1 bound to the particles and protected them from PBS.

DLS (fig.12B) giving a large variation in the size and the data of some PGB1 concentrations were unacceptable. It might be due to the aggregation of GNPs as these experiments had been done after one year from previous measurements and the stability of the particles has to be checked.

A.



B.

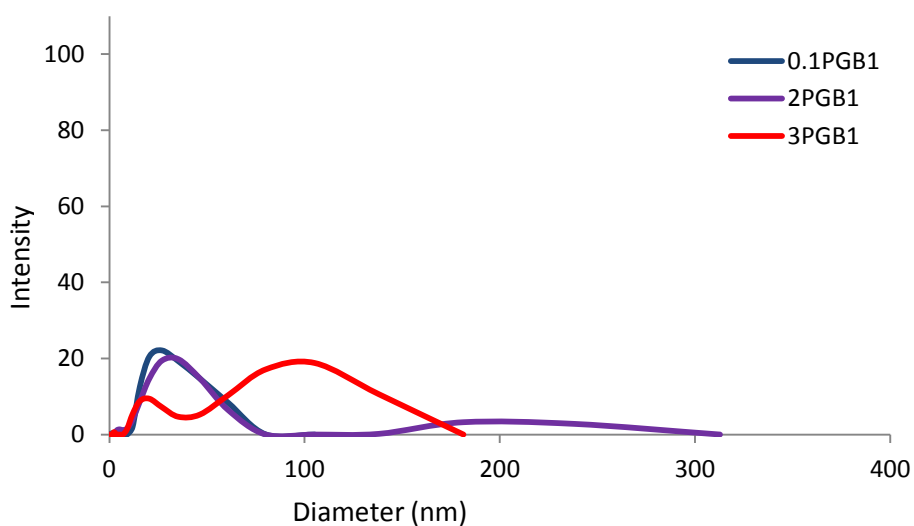


Fig.12: (A) Disc centrifuge and **(B)** DLS measurements of different PGB1 concentrations in PBS.

3.8 Stability of GNPs after one year

After one year, the stability of GNPs in water and PBS was measured again using DLS and disc centrifuge (fig.13). In the DLS, the data were unacceptable giving a large variation in the size so the machine could not calculate an average size. In the disc centrifuge the data were acceptable (figure 1), particles in water has a single peak with maximum intensity around 18.14nm but in PBS, intensity decreases and the particles aggregated giving a broad polydisperse peak. DLS has higher sensitivity to the aggregates and impurities than disc centrifuge so ,may be that is why the GNPs after one year couldn't be measured by DLS although it was measured with the same method one year before ^[18] .

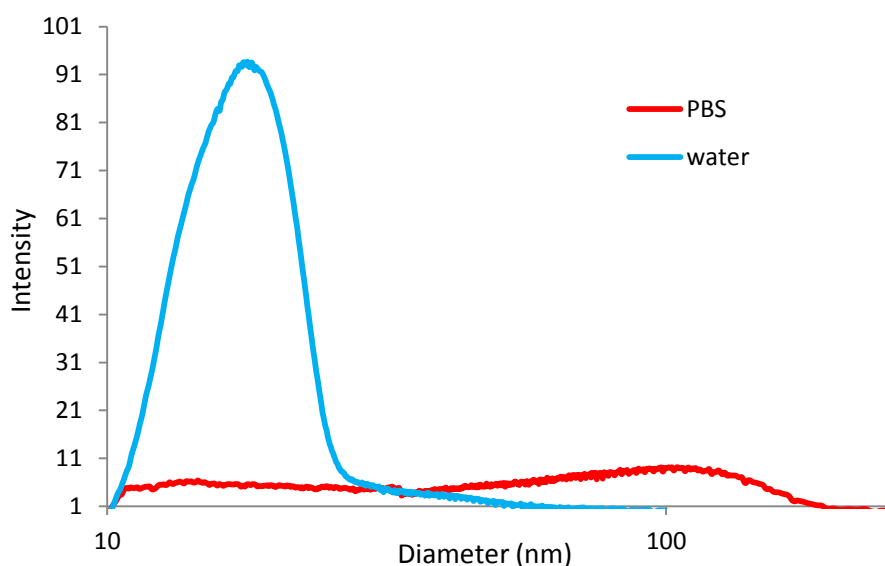


Fig.13: Disc centrifuge measurement of GNPs in water (cyan) and PBS (red) after one year from DLS measurement (fig.1).

Other GNPs were used to check that the aggregation of particles was actually due to IgG content in cow serum and not due to the aggregation of the particles itself. The size of the particles in water and PBS was measured by disc centrifuge and DLS. Both of them gave acceptable results. The size of the particles in water was 23 nm measured by disc centrifuge while in the DLS was 26 nm and this acceptable as DLS measured the hydrodynamic diameter so it was always more than the diameter measured by disc centrifuge. The size of the particles dissolved in PBS was 100 nm according to disc centrifuge data while in DLS there were two peaks one around 539 nm and 4774.3 nm.

Different concentrations of PGB1 with 2.5mM GNPs dissolved in PBS were incubated for 30 minutes and the size was measured by disc centrifuge and DLS. In the disc centrifuge, a big difference in the size between GNPs dispersed in PBS and the particles with different

PGB1 concentrations (0.1, 0.5, 1, 2 and 3 mg/ml). The size of the particles decreased by adding PGB1 and this was agreed with the data from the other gold particles. By increasing PGB1 concentration, just a small shift in the peaks towards bigger size could be seen but there was no aggregation. The same with DLS measurements, also small increase in the size but at 3 mg/ml PGB1 the size decreased which might mean that all the particles totally covered by PGB1 and no particle exposed to PBS so, there was no any small aggregation.

The aggregation of GNPs conjugated to 1mg/ml PGB1 and different cow serum concentrations (1, 5, 10, 30 and 50%) in PBS was measured using disc centrifuge (fig.14) and DLS. The DLS data were unacceptable while the particles size could be measured by disc centrifuge. At 1% cow serum, a small single peak appeared at 16 nm which was nearly the same as the peak of the control sample (1mg/ml PGB1 with 2.5mM GNPs). The reason for that was the low concentration of cow serum. At 5% cow serum, another peak appeared at 78 nm which an indication about the aggregation of the particles. The aggregation increased by increasing serum concentration and the biggest aggregation was at 50 % cow serum (blue curve, fig.15)

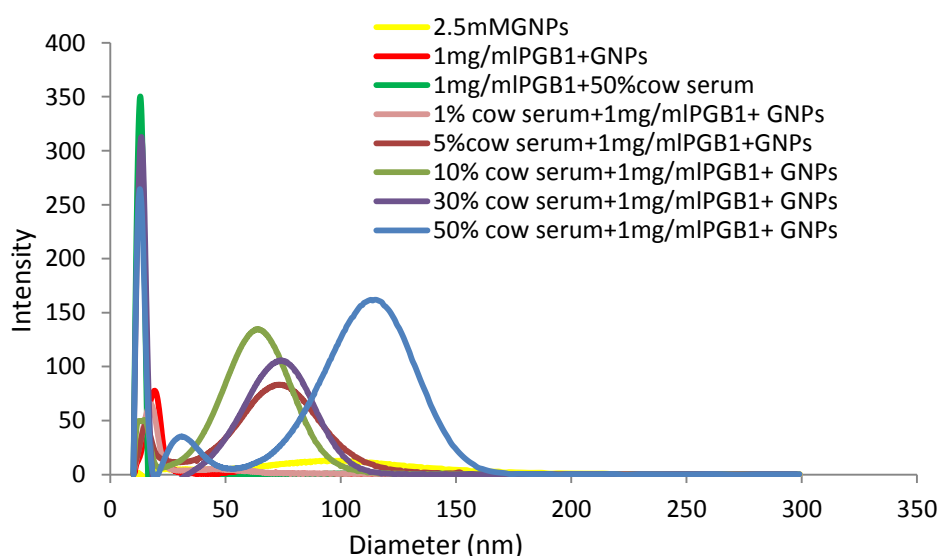


Fig.14: Disc centrifuge measurements of 2.5mM GNPs with 1 mg/ml PGB1 and different cow serum concentrations (1, 5, 10, and 30%).

Carboxylated GNPs

Conjugation of carboxylated GNPs with PGB1 had been tried and incubated with different concentrations of IgG, cow and calf serum. Then, the size of the conjugated particles was measured by DLS and disc centrifuge. It seems that this system is complicated and no significant difference between conjugated and unconjugated carboxylated GNPs could be detected.

Biotinylated GNPs

There was no difference between conjugated and unconjugated streptavidin particles with different concentrations of cow serum could be seen. It might be that there was no interactions between streptavidin and cow serum. To study the behavior of streptavidin, it was diluted 10 times (0.05 mg/ml) and labeled with Alexa flour 488. The fluorescence was measured using fluorimeter but the signal was very noisy as the streptavidin concentration was very low.

4. Conclusions

- The water stabilizes the particles while PBS aggregates them.
- These aggregations can be controlled by conjugating PGB1 protein to the particles before adding PBS as PGB1 binds to GNPs surfaces.
- PGB1 conjugated to particles cannot be seen in SDS-PAGE.
- PGB1 binds to IgG of cow serum.
- Low cow serum concentration aggregates GNPs more than higher concentrations and more than calf serum.
- Aggregations of GNPs depends on IgG concentration, i.e. low concentration causes high aggregation, medium concentration causes medium aggregations, high concentration causes low aggregations.
- The high aggregated samples cannot be measured by DLS.
- The size of particles using DLS is bigger than disc centrifuge as it measures hydrodynamic diameter.
- The stability of GNPs is changing over time (particles aggregated after one year).
- Carboxylated and biotinylated GNPs are complex systems to work with.

5. Future work

- Study the effect of GNPs size on the protein corona.
- Run gel for PGB1 conjugated 2.5mM GNPs with different concentrations of cow and FCS.
- More studies on carboxylated GNPs.
- Increase the streptavidin concentration used for fluorescence labelling by Alexa flour 488.

6. References

1. Braydich-Stolle, L., et al., *In Vitro Cytotoxicity of Nanoparticles in Mammalian Germline Stem Cells*. Toxicological Sciences, 2005. **88**(2): p. 412-419.
2. Chou, L.Y.T. and W.C.W. Chan, *Fluorescence-Tagged Gold Nanoparticles for Rapidly Characterizing the Size-Dependent Biodistribution in Tumor Models*. Advanced Healthcare Materials, 2012. **1**(6): p. 714-721.
3. De Jong, W.H. and P.J.A. Borm, *Drug delivery and nanoparticles: Applications and hazards*. International Journal of Nanomedicine, 2008. **3**(2): p. 133-149.
4. Perrault, S.D., et al., *Mediating Tumor Targeting Efficiency of Nanoparticles Through Design*. Nano Letters, 2009. **9**(5): p. 1909-1915.
5. DeLong, R.K., et al., *Functionalized gold nanoparticles for the binding, stabilization, and delivery of therapeutic DNA, RNA, and other biological macromolecules*. Nanotechnology, Science and Applications, 2010. **3**: p. 53-63.
6. Das, M., et al., *Review on gold nanoparticles and their applications*. Toxicology and Environmental Health Sciences, 2011. **3**(4): p. 193-205.
7. Tenzer, S., et al., *Nanoparticle Size Is a Critical Physicochemical Determinant of the Human Blood Plasma Corona: A Comprehensive Quantitative Proteomic Analysis*. ACS Nano, 2011. **5**(9): p. 7155-7167.
8. Rahman, M., et al., *Nanoparticle and Protein Corona*, in *Protein-Nanoparticle Interactions*. 2013, Springer Berlin Heidelberg. p. 21-44.
9. Albanese, A., P.S. Tang, and W.C.W. Chan, *The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems*. Annual Review of Biomedical Engineering, 2012. **14**(1): p. 1-16.
10. Nel, A.E., et al., *Understanding biophysicochemical interactions at the nano-bio interface*. Nat Mater, 2009. **8**(7): p. 543-557.
11. Sonavane, G., K. Tomoda, and K. Makino, *Biodistribution of colloidal gold nanoparticles after intravenous administration: Effect of particle size*. Colloids and Surfaces B: Biointerfaces, 2008. **66**(2): p. 274-280.
12. Koutsopoulos, S., et al., *Adsorption of Trypsin on Hydrophilic and Hydrophobic Surfaces*. Langmuir, 2007. **23**(4): p. 2000-2006.
13. Salvati, A., et al., *Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface*. Nat Nano, 2013. **8**(2): p. 137-143.
14. Gallagher, T., et al., *Two Crystal Structures of the B1 Immunoglobulin-Binding Domain of Streptococcal Protein G and Comparison with NMR*. Biochemistry, 1994. **33**(15): p. 4721-4729.
15. Dobrovolskaia, M.A., et al., *Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles*. Nanomedicine : nanotechnology, biology, and medicine, 2009. **5**(2): p. 106-117.
16. Cukalevski, R., et al., *IgG and fibrinogen driven nanoparticle aggregation*. Nano Research, 2015. **8**(8): p. 2733-2743.
17. Sartor, M., *DYNAMIC LIGHT SCATTERING* University of California San Diego.
18. Bell, N.C., C. Minelli, and A.G. Shard, *Quantitation of IgG protein adsorption to gold nanoparticles using particle size measurement*. Analytical Methods, 2013. **5**(18): p. 4591-4601.

19. *Introduction to Differential Sedimentation*, in *Analytical Ultracentrifugation: Techniques and Methods*, D.J. Scott, S.E. Harding, and A.J. Rowe, Editors. 2005, The Royal Society of Chemistry. p. 270-290.
20. Price, N. and J. Nairn, *Exploring Proteins: a student's guide to experimental skills and methods*. 2009: OUP Oxford.
21. Jana, N.R., L. Gearheart, and C.J. Murphy, *Seeding Growth for Size Control of 5–40 nm Diameter Gold Nanoparticles*. *Langmuir*, 2001. **17**(22): p. 6782-6786.
22. Lindman, S., et al., *In vivo protein stabilization based on fragment complementation and a split GFP system*. *Proceedings of the National Academy of Sciences of the United States of America*, 2010. **107**(46): p. 19826-19831.
23. *CPS Disc Centrifuge Operating Manual*
24. <http://www.cytodiaagnostics.com/store/pc/Covalent-Conjugation-of-Proteins-to-Carboxylated-Gold-Nanoparticles-d10.htm>.
25. <https://tools.thermofisher.com/content/sfs/manuals/mp00143.pdf>.
26. Albanese, A. and W.C.W. Chan, *Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity*. *ACS Nano*, 2011. **5**(7): p. 5478-5489.

Appendix I

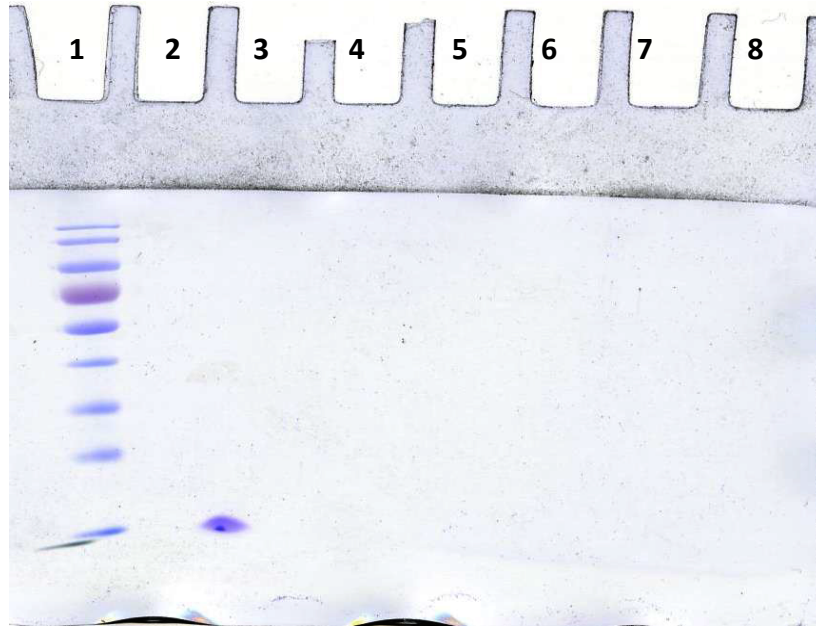


Fig.15:SDS-PAGE of different PGB1 concentration with 0.113 mg/ml GNPs.(1) Ladder, (2) 3mg/ml PGB1, (3) GNPs+0.1mg/ml PGB1,(4) GNPs+0.5mg/ml PGB1, (5) GNPs+0.1mg/ml PGB1, (6) GNPs+1mg/ml PGB1, (7) GNPs+0.1mg/ml PGB1 and (8) GNPs+0.1mg/ml PGB1.

Appendix II

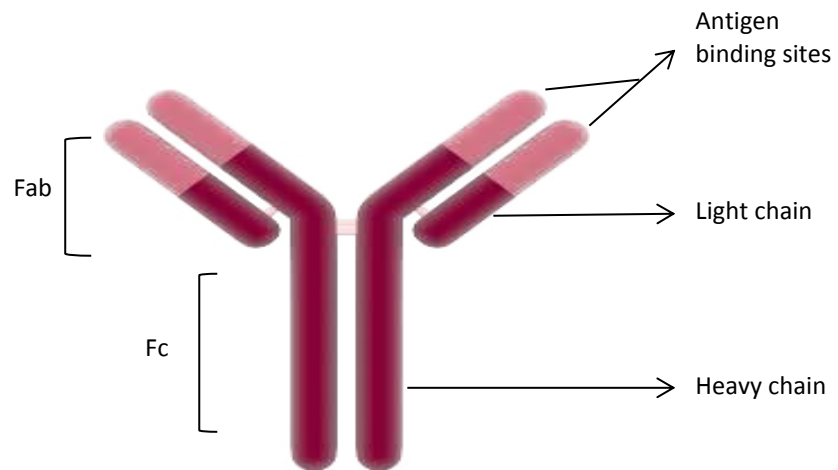


Fig.16: The general structure of IgG.

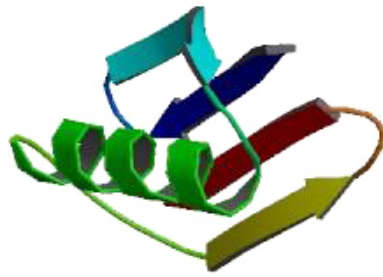


Fig.17: PGB1 structure from pdb viewer with ID 1pgb.

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