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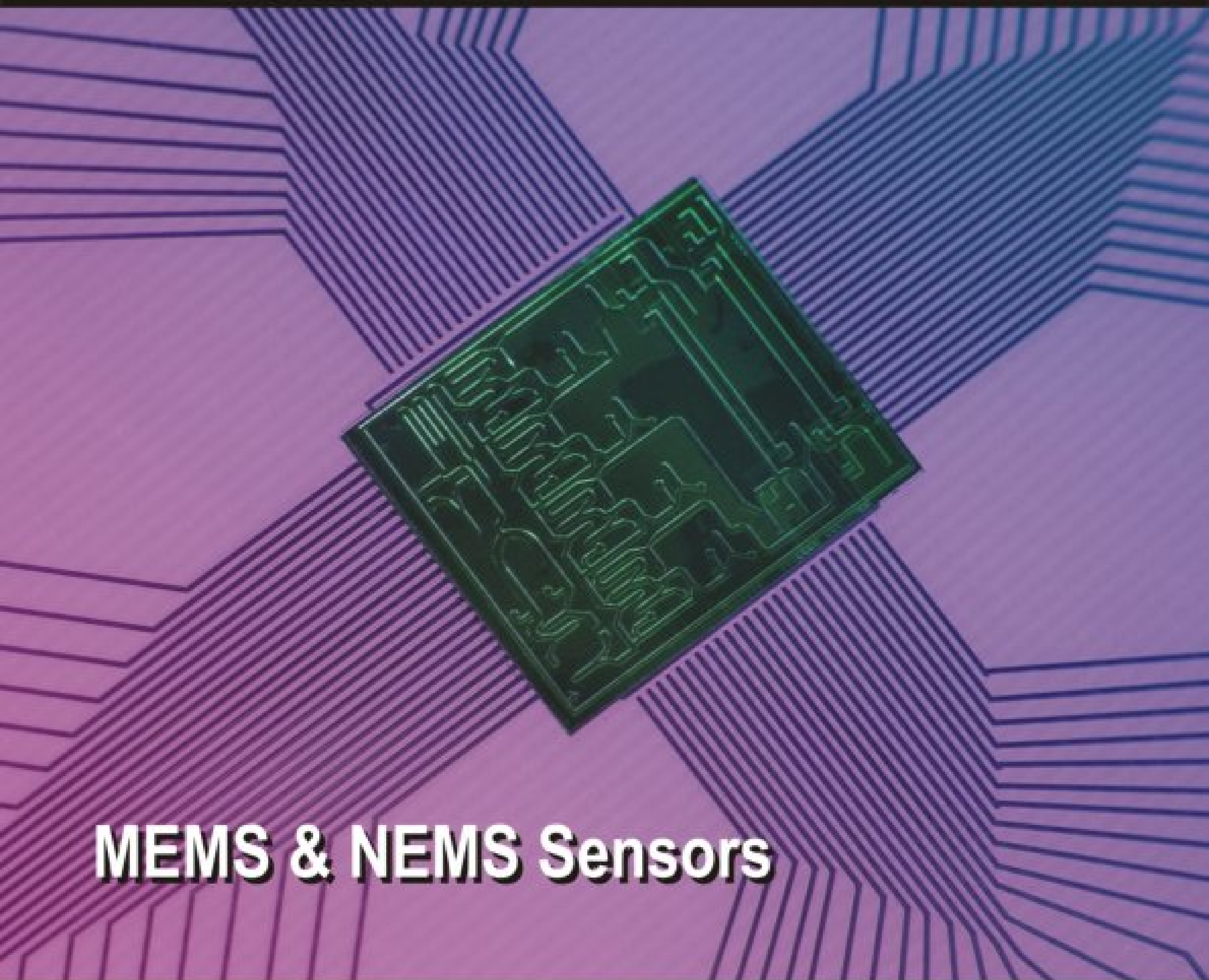

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Ultralow Detection of Bio-markers Using Gold Nanoshells

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Abstract: Gold nanoparticles (AuNPs) as identification markers or labels can be designed for detection of antigens, analytes, biomarkers, or viral particles, in small sample volumes and concentrations, in a very sensitive and specific environment. The focus of this project is to improve the detection of antigen/biomarkers/viral vectors using gold nanoshells (GNS), which results in a change in the surface plasmon resonance (SPR). The assay used in this project is based on aggregation of GNS in presence of antigen or viral vectors. GNS are coated with thiolated–poly(ethylene) glycol–amine (HS-PEG-NH_2)-conjugated Protein G (ProG) and Goat anti-rabbit antibody. Our data shows that fully assembled nanoparticles (GNS-PEG-ProG-Anti-rabbit) in presence of antigen, rabbit IgG (Rb IgG), allow detection events at concentrations in the ng/ml to pg/ml range. The lower detection limit of this improved assay was 0.5 pg/mL within 10 mins. This shows a three order of magnitude improvement over that previously reported. *Copyright © 2012 IFSA.*

Keywords: Gold nanoshells, Detection, Protein G, Enhanced detection limit, Bio-assay.

1. Introduction

Astronauts experience lengthy and repeated exposure to radiation such as heavy ions, solar particle events [1], and galactic cosmic rays (GCR) during extended missions. These have been linked to the initiation and progression of carcinogenesis, cell apoptosis, and tissue degeneration by inducing DNA damage and subsequent mutations [2, 3]. The use of gold nanoparticles as identification markers or labels can allow for detection of antigens, analytes, biomarkers, or viral particles, in small sample volumes and low concentrations, in a very sensitive and specific environment [4]. By optimizing gold nanoparticles to selectively bind to markers expressed after these events, a sensitive, portable low weight platform for detection of cellular damage can be developed which would allow proactive decisions prior to return to earth for full diagnostics. In this paper, we focus on a system capable of rapid detection on variety of analytes/biomarkers using gold nanoshells (GNS), which can be coupled with Lab-on-chip device [5] as a portable diagnostic tool.

Gold nanoparticles (AuNPs) have had a tremendous presence in applications for immunoassay [6], detection markers, drug delivery [7, 8], contrast enhancement, hyperthermia for tumor therapy [9], and combined with imaging and therapy [10]. The use of AuNPs as identification markers or labels, allows for detection of antigens, analytes, biomarkers, or viral particles, in small sample volumes and concentration in a very sensitive and specific environment [4]. In this paper, we focus on a system capable of rapid detection on variety of analytes using GNS, but can also be coupled with Lab-on-chip [5] for a portable diagnostics tool. This system could be used in diagnosis of respiratory viruses, cancer, HIV, hepatitis B, C and E, Herpes simplex virus and bioterrorism agents in the modern era.

Immunoassays play an important factor in clinical diagnostics, which are dependent on recognition of target antigens by specific antibodies. However, assays such as the enzyme linked immunosorbent assay (ELISAs) are not convenient when fast response with accuracy is required [11]. ELISA based systems have two key disadvantages, 1) that they comprise of time consuming steps, including: sample preparation, purification, incubation, and multiple rinsing steps, and 2) increases usage of materials necessary to perform these assays [6, 12]. To overcome these problems, a homogeneous immunoassay was developed using GNS tethered with Protein G and antibody. The immunoassay is based on polyclonal antibody-Protein G-GNS aggregation upon interaction with an analyte present in solution; this allows particles to bind to multiple analyte molecules, which leads to these particles linking with other particles, these binding events changes the resonance profile associated with individual nanoparticles allowing sensitive detection [6].

GNS are tunable spherical nanoparticles, composed of a dielectric core covered by a thin gold shell, with diameters ranging from 10 to 200 nm (Fig. 1) [13]. GNS have optical, chemical and physical properties, which make them an ideal candidate for enhancing cancer detection, cancer treatment, cellular imaging and medical biosensing. The geometry of the GNS determines the optical properties.

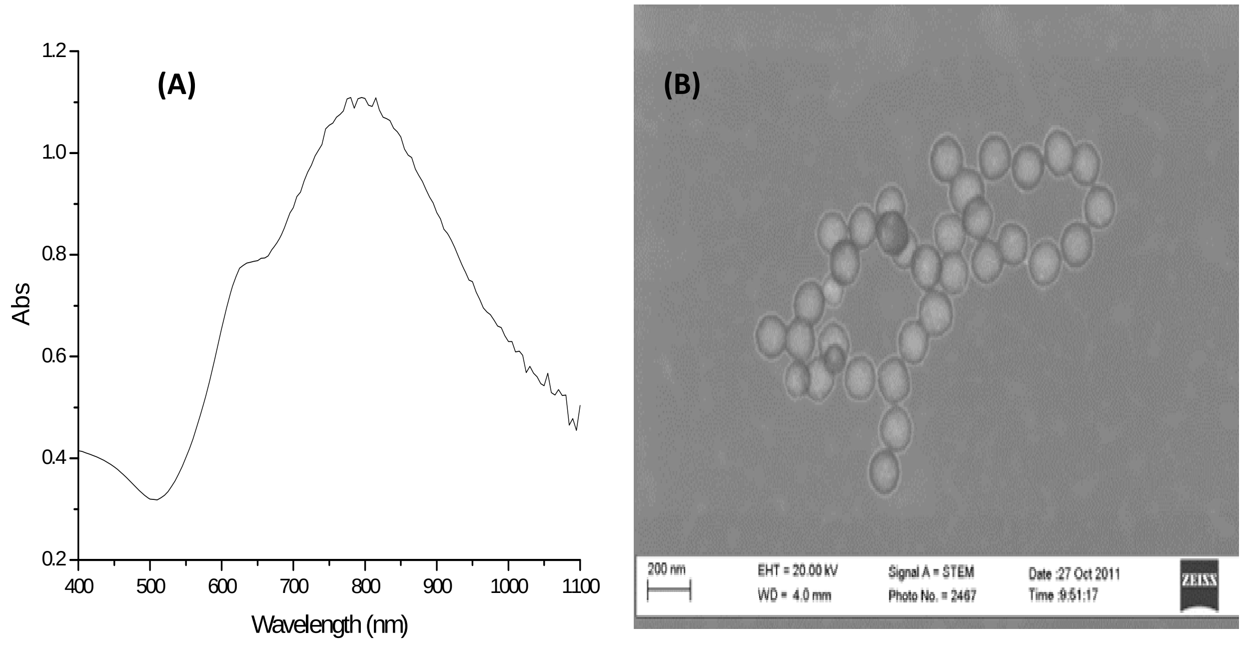

Fig. 1. Spectra profile (A) image of the GNS that were fabricated with radius of ~74 nm and core of ~60 nm and STEM image (B) to confirm the synthesis.

The ratio of shell thickness to core diameter places the peak resonance; while the overall size determines absorbing vs. scattering properties of the particle. Furthermore, the inert gold surface provides several benefits, including affinity for thiol groups, which simplifies conjugation to antibodies or other biomolecules for both active tumor targeting and biosensing applications.. The former property is important for chemical conjugation of antibodies, or other biomolecules for both active tumor targeting and bio sensing applications on the surface of the particle [13]. This system has the capability to be utilized in diagnosis of various viruses, and bioterrorism agents in the modern era [10]. Nanoshells can be fabricated with tunable optical properties, absorbing or scattering, form visible through infrared spectrum [9]. The principle by which this assay works involves a change in the resonance of the particles when they are linked and are in close proximity to each other. If a linker causes multiple particles to bind, the absorbance at the peak is greatly reduced. In this assay, polyclonal antibodies on the nanoparticles' surface bind to multiple regions of the antigen, causing an aggregation effect of the nanoparticles when antigen is present. Biomarker detection via nanoshell aggregation has been demonstrated by Hirsch et. al. in 20 % whole blood with detection levels down to 0.8 ng/ml in 30 minutes [6]. By carefully designing the tethering mechanism to orient the antibody for maximum activity, we hypothesized that these levels of detection could be greatly increased.

Protein G (ProG) is a protein that has the ability to specifically bind to antibody by F_c domain region, which are found on the surface of a variety of staphylococci and streptococci [5, 14]. Using protein and gene sequence analysis it was reported that ProG is similar to Protein A (ProA) [15]. ProG binds human IgG of all four subclasses and also to mouse monoclonal IgG, the affinity constant is higher than of ProA [5]. Therefore, it is a great candidate that can be used as a cofactor to capture antibody on the surface of GNS to allow for maximum binding activity to its antigen. Recombinant ProG is a single chain protein that lacks free thiol groups to bind to gold surfaces. The physical adsorption of ProG on GNS leads to random coating of ProG with potential loss of IgG binding activity. Therefore more oriented coupling of antibody can occur using the capture of the genetically or chemically modified ProG bound on gold surface [1, 16-19].

The system reported here, uses a modular conjugation assembly based on ProG to create an antibody-ProG-GNS particles. This particle system was exposed to samples of known analyte concentrations ranging from ng/mL to pg/mL levels and demonstrated optical changes in the spectra over time, permitting measurements at statistically differences from zero with detection levels of 0.5 pg/ml within only 5-10 minutes.

2. Methods and Material

2.1. GNS Synthesis

Monodispersed silica nanoparticles (~7 %, dia. ~ 120 nm) were prepared by the Stöber method. Briefly, 14 mL of Ammonium Hydroxide (NH_4OH, 30 %, Sigma) was mixed with 225 mL of Absolute Ethanol (C_2H_5OH, 99.5 %, Sigma), and 7.5 mL of Tetraethyl Ortho-silicate (TEOS, 99.999 %, Sigma) solution was added into the mixture and stirred overnight. Next, silica nanoparticles were functionalized amino groups by 3-Aminopropyl Trimethoxysilane (APTES, 99 %, Sigma). About 200 mg silica nanoparticles were dispersed in 45 mL 90 % ethanol solution which contain 4 % APTES in a 50 mL plastic tube. The tube was placed in a sonication bath at 75 ^{o}C for 4 hr, with sonication every 30 mins. Then the sample was left on rotator at room temperature (RT) for 20 hrs. After amino-silanization, silica particles were separated and washed with an ethanol solution via centrifugation at 1000 G for 20 minutes and resuspension of the pellet in neat alcohol.

Gold colloid was prepared to a size of 2-4 nm using Duff et al method [20], and aged 3 weeks at 4 °C. The colloid sample was then concentrated 10X through rotary evaporation and mixed with the aminated silica nanoparticles. This allows small gold colloid to attach to silica nanoparticles surface, to act as nucleation sites for the reduction step, to allow formation of a contiguous shell of gold. The final step, growing the gold shell, was accomplished by the reduction of gold from Hydrogen Tetra-chloroaurate (III) Hydrate ($HAuCl_4$, 99.99 %), in the presence of Formaldehyde (HCOH, 37 %). $HAuCl_4$ reduction solution was prepared by adding 3.0 mL of 1.0 wt % $HAuCl_4$ to 200 mL Potassium Carbonate (K_2CO_3, 99.7 %) solution (50 mg of K_2CO_3 in 200 mL DI water), and aged for two days before use. The GNS were analyzed using Cary 80 BIO UV Vis spectrophotometer, ZetaPals, and STEM.

2.2. PEG-ProG Conjugation

Thiol–poly(ethylene) glycol–amine (SH-PEG-NH_2, 1000 Da, LaysanBio) and Protein G′ (ProG) from Streptococcus sp (P4689, Sigma), were dissolved at 1 mg/mL in PBS and stored separately. Next, NH_2-PEG-SH and EDC were mixed with ProG at a mole ratio of 20:20:1 and reacted overnight at 4 °C. The sample was then purified using NanoSep Centrifuge (OD010C33, VWR) and stored at -20 °C for later use.

2.3. GNS-PEG-ProG-Anti-Rb Conjugation

PEG-ProG conjugate were added onto GNS, in Kcarb, at a targeted mole ratio of 5000:1, and reacted at 4° C for 4 hrs with agitation every ~30 mins. Next, Goat Anti-Rabbit (R2004, Sigma) at 1 mg/mL, was added to GNS-PEG-ProG conjugate at mole ratio of 5000:1 and reacted same as PEG-ProG. To backfill any remaining unspecific, "empty", sites on the particle, 10 µL of 10 µM PEG-SH (5000 Da, LaysanBio) was added and reacted overnight. Prior to adding Anti-Rb or PEG-SH, the GNS sample was split into 5 mL aliquots and centrifuged at 650 g for 7 mins. The supernatant was removed immediately and aliquots were resuspended in 5 mL Kcarb.

2.4. ELISA on GNS to Determine the Number of Anti-Rb on Surface

An ELISA was carried out using a modification of the Lowery-Gobin quantification method. [21] Briefly, GNS-PEG-ProG-Anti-Rb conjugate were washed and resuspended in PBS; followed by incubation with Anti-Goat HRP (horseradish peroxidase labeled, A9452, Sigma) for 1 hr at RT. For a negative control, GNS blocked with PEG-SH was used. Next, the samples are blocked with 3 % PBSA, for preventing unspecific binding reaction and washed afterwards to remove any unbound or excess antibody. Final GNS conjugates were then reacted with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate with fresh hydrogen peroxide for 15 mins; the reaction was stopped with H_2SO_4. Finally, the samples were read using BioTek ELx800 plate reader at 450 nm (Fig. 2).

2.5. Detection of Antigen/IgG Complex

GNS-PEG-ProG-Anti-Rb were fabricated as mentioned above and blocked with PEG-SH. The GNS-conjugate were washed and resuspended in DI water prior to use. Next, IgG from rabbit serum (I5006 lyophilized powder, Sigma) was dissolved and diluted to 50, 5, 0.5, 0.05, and 0.005 ng/mL in PBS and stored at 4° C until needed. GNS-conjugate were placed in a quartz cuvette and diluted to 1 OD using DI water, PBS, or Serum (FBS), and with 100 µL of the IgG, at different concentration as mentioned above, to bring the final concentration of IgG to 5, 0.5, 0.05, 0.005, and 0.0005 ng/mL (0.5 pg/mL); while remaining the concentration of GNS at 1 OD. Samples were mixed and spectral scans of each sample were collected every 5 mins for 1 hr (also referred to as kinetic scans).

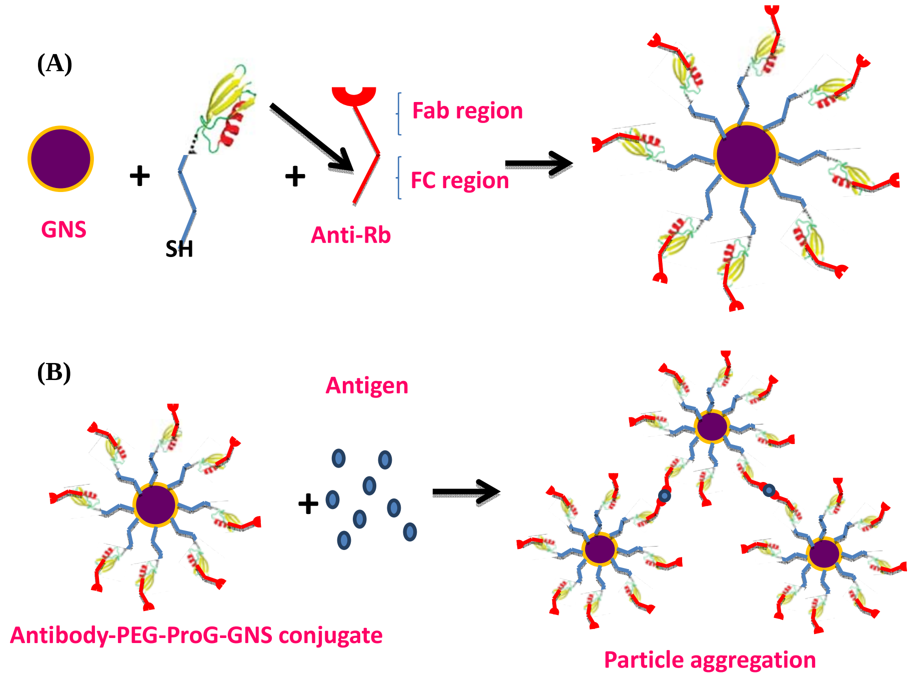

Fig. 2. A step by step schematic of the GNS-PEG-ProG-Anti-Rb conjugation (A) and the interaction of GNS with antigen leading to particle aggregation.

3. Results and Discussion

3.1. Assessment (or Analysis) of GNS-PEG-ProG-Anti-Rb Conjugation

After each step of the assembly, NIR peak, dynamic light scattering (DLS), and zeta potential (charge) measurements were taken to assess size and surface properties. From Table 1, we can see the size change from 195.2 nm to 241.2 nm, which is due to the addition of PEG-ProG, Anti-Rb, and PEG-SH.

Table 1: Characteristic parameter shifts by the ProG-Ab conjugated GNS. Bare and conjugated GNS were suspended in Kcarb and DI water, respectively.

GNS	Spectra	Size (in Kcarb, nm)	Zeta (in Kcarb, mV)	Size (in DI, nm)	Zeta (in DI, mV)
Bare	814	195.2	-61.6	196.3	-32.7
ProG	816	204.4	-57.8	209.6	-33.2
ProG-Ab	823	239.6	-29.5	-	-
ProG-Ab-PEG	818	241.2	-27.2	239.2	2.09

3.2. Quantification of GNS to Determine the Number of Active Antibodies on Particle Surface Using an ELISA

Due to the ability of ProG to capture multiple antibodies, there was a significantly higher amount of antibodies bound on the surface of fully assembled GNS compared previously reported data and to PEG controls. Prior to running the ELISA, GNS-PEG-ProG-Anti-Rb samples were assembled using different molecular ratios of antibody to particles: 1000:1, 3000:1, and 5000:1. From Fig. 3, using the 5000:1 ratio resulted in approximately 311 antibodies per GNS. The antibody density reported here is twice that obtained by methods reported previously ($p < 0.00001$) [21].

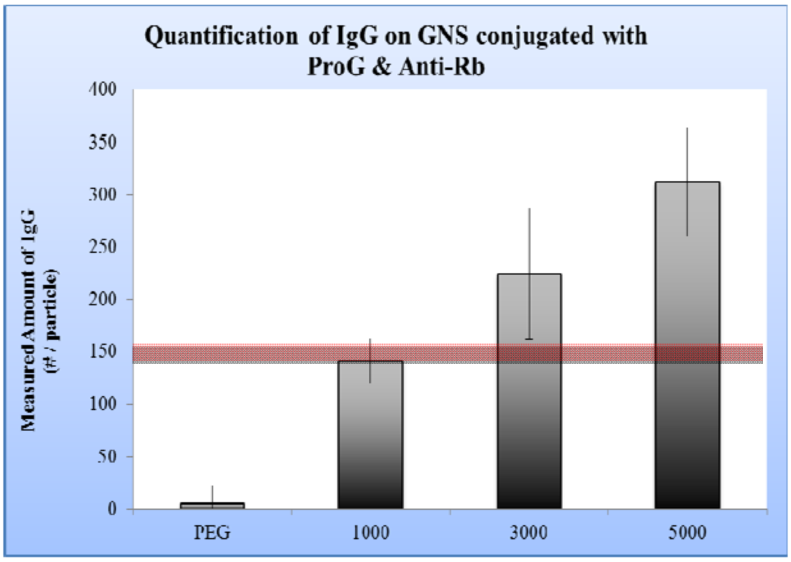

Fig. 3. ELISA-based Lowery-Gobin quantification method to determine the number of antibodies on GNS with different antibody to GNS ratios, red bar represents the amount of antibodies on GNS reported previously.

3.3. Detection of Analyte Complex

These initial studies are focused on analyte detection in saline solutions, using GNS-PEG-ProG-Anti-Rb, has the capability to quantitatively detected analytes at various concentrations (5-0.0005 ng/mL) within 10 mins of the kinetic scan, in all controls as seen in Fig 4 .

The analyte detection assay was performed using kinetic scans for five different analyte concentrations in PBS; Fig 4 indicates the percent decrease in absorbance of GNS, from t=0 to 65 mins, in presence of analytes, compared to analyte-free samples which shows little to no aggregation. The aggregation or formation of multi-mer particle complexes was measured at 820 nm. With increasing concentration of the analytes, the assay demonstrates rapid aggregation kinetics at early times. Fig 5, STEM images were taken of two samples after the assay was competed to show rapid aggregation. All five analytes concentrations were statistically different compared to control samples after 10 min of the kinetic scan, $p<0.05$.

In order to detect the analytes in serum, the GNS conjugate, GNS-PEG-ProG-Anti-Rb, was fabricated as mentioned above and blocked with PEG-SH. Next, GNS-conjugate were washed and resuspended in 10 %, 30 %, or 50 % FBS prior to use. The analyte, IgG from rabbit serum, was dissolved and diluted to 50, 5, 0.5, 0.05, and 0.005 ng/mL in 10 %, 30 %, or 50 % FBS and stored at 4° C until needed. GNS-conjugate were placed in a quartz cuvette and diluted to 1 OD using 10 %, 30 %, or 50 % FBS, and with 100 μL of the IgG, at different concentration as mentioned above, to bring the final concentration of IgG to 5, 0.5, 0.05, 0.005, and 0.0005 ng/mL (0.5 pg/mL); while remaining the concentration of GNS at 1 OD. Samples were mixed and spectral scans of each sample were collected every 5 mins for 1 hr. However, none of the serum studies were successful. No significant absorbance drop was detected, nor particle dimer formation; because serum is composed of albumin/proteins and other impurities steric hindrance may be dominating. The highest affinity to protein G was found for serum albumins from rat, man, and mouse; while a medium affinity was found form rabbit, cow, hen, and horse [22]. This affinity binding profile along with steric hindrance created a challenge for the assay, not only was the antigen being blocked, but with the low concentration of antigen makes it impossible to bind to the antibody.

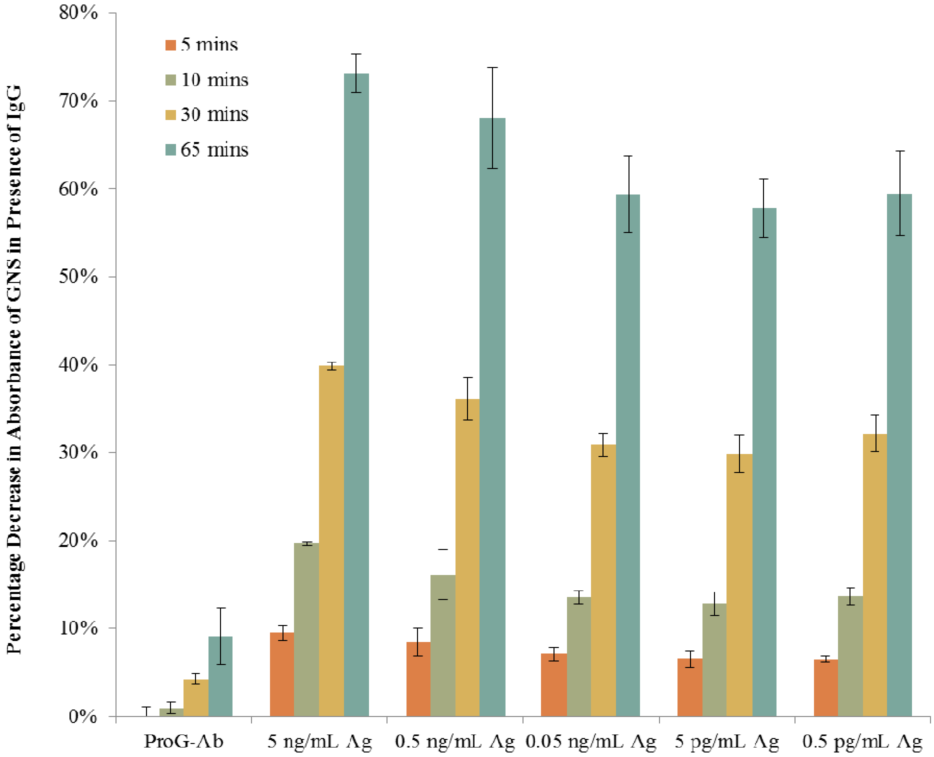

Fig. 3. Percentage decrease in absorbance of GNS in presence of Analyte/IgG complex. From left to right: ProG-Ab, a negative control to show the stability of GNS-PEG-ProG-Anti-Rb conjugate. ProG-Ab+5...0.0005ng/mL (0.5 pg/mL), the sample incubated with analyte and kinetic change was observed over an hour.

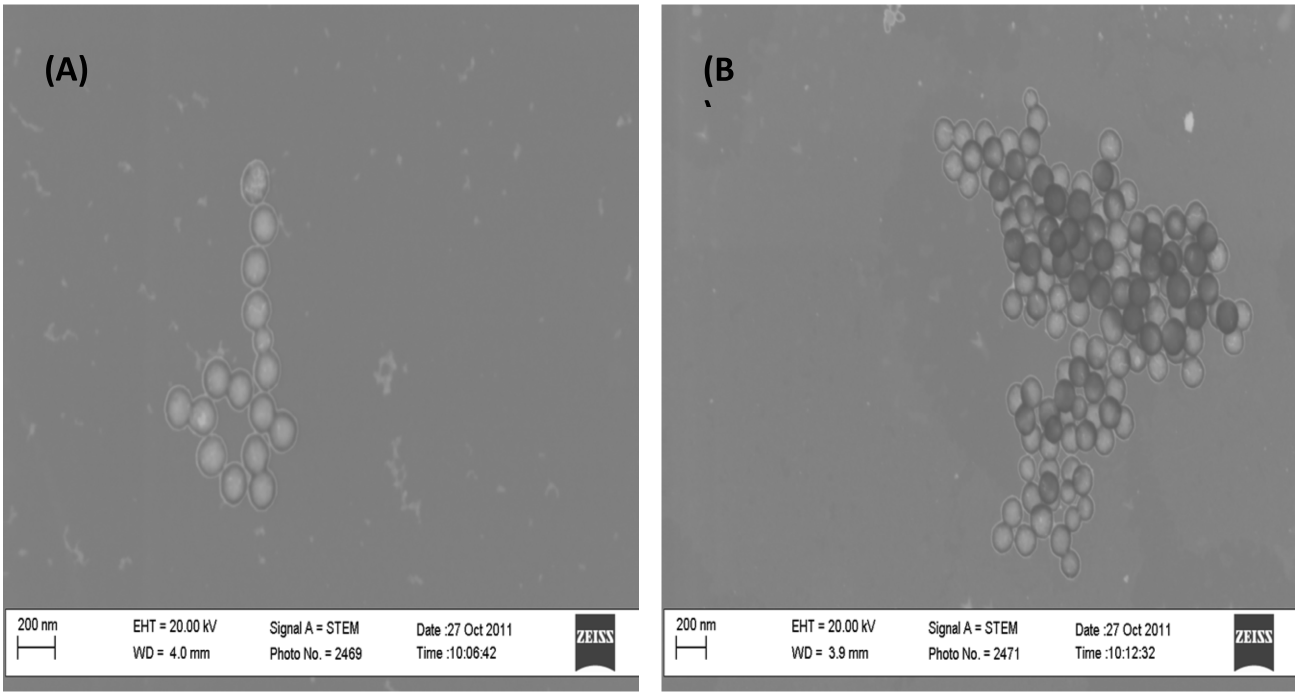

Fig. 4. STEM images taken of (A) ProG-Ab (sample without antigen) and (B) ProG-Ab with 5 ng/mL of antigen after the liquid detection assay.

To overcome this issue, we utilized the same GNS conjugate as tags or labels to allow for detection of antigens or viral particles in small sample volumes in a very sensitive and specific environment. This can be used as an advanced early detection system, which enables accurate readings using lab-on-chip or microfluidic device coupled with sandwich an enzyme-linked immunosorbent assay (ELISA). We propose a system, which uses ELISA steps, and silver stain enhancement with targeted GNS for cost effective diagnostics. The field of medical virology has seen numerous technological advancements in the past few decades for diagnosis of viral infections. ELISA was introduced in laboratories during the 1980s. The assay was designed to detect antibodies, specific immunoglobulins (IgG, IgM, IgA) to viruses or viral antigens/particles. A standardized sandwich-ELISA system using silver stain enhancement with targeted gold nanoparticles for cost effective diagnostics can be developed for detecting viral nanoparticles. The analyte detection system is very quick, sensitive, and has more manipulability when certain nanoparticles are used [23].

Goat-Anti-Rabbit-IgG-Biotin (B8895, Sigma) was incubated on a Streptavidin (SuperStreptavidin – SMS, Arrayit) coated glass slides for 1.5 hrs with multi-well microarray. Next, the wells were blocked with SuperStreptavidin Blocking buffer (SBB, Arrayit) for 1 hr, followed by incubation of IgG from rabbit serum at various concentrations, in PBS or FBS for 1 hr. The GNS conjugate, GNS-PEG-ProG-Anti-Rb, was fabricated as mentioned above and blocked with PEG-SH, washed and stored in PBS or FBS. Next, GNS-conjugate was incubated for 30 mins to wells with IgG from rabbit serum, a PEG-GNS and blank controls were also used. Sample wells were then washed and a silver stain was performed; slides imaged and the grayscale intensity measurements were analyzed using Nikon NIS viewer as seen on Fig 6.

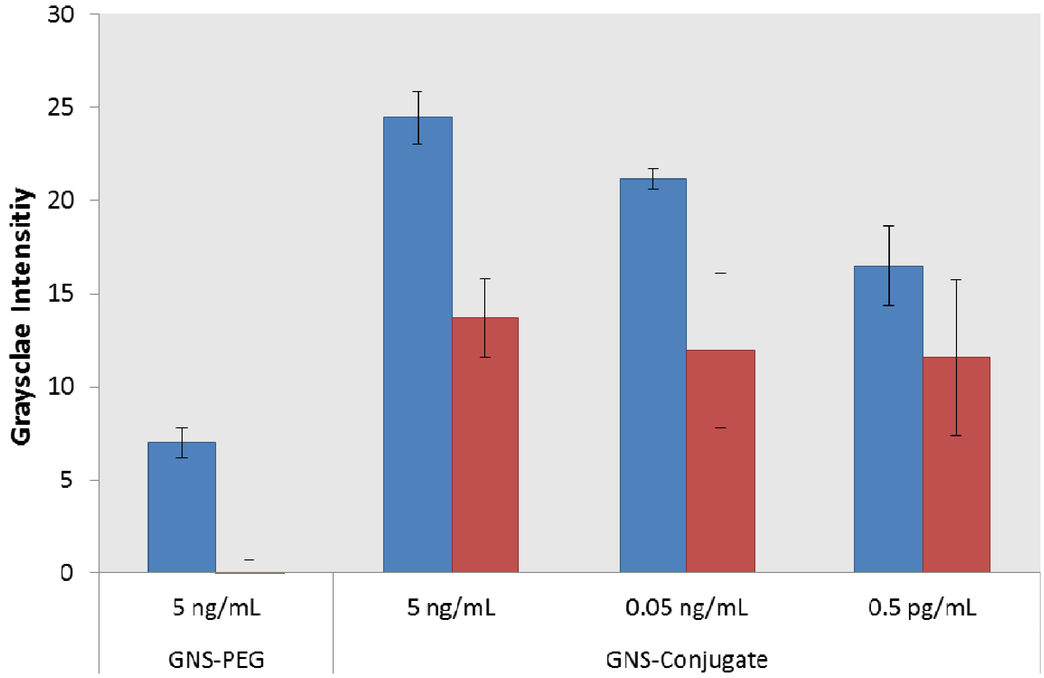

Fig. 5. Grayscale intensity results after ELISA was completed. Blue and Red bars represents samples incubated in PBS and FBS, respectively.

ELISA based systems have been used in medical virology for detection of antibodies, immunoglobulins to viruses, viral antigens or nanoparticles. Traditional ELISA based detection systems have several limitations. First, antibodies used are generally laid on various types of surfaces in a random orientation, which makes the protein markers, viruses, viral antigens and nanoparticles inaccessible to antibodies, which eventually return skewed data. Second, low ratios of antibodies to viral vectors may result in limited sensitivity. Third, a viral detector with many modifications can lead to inefficiency of its function and it is a time consuming process. By slight modification to standard ELISA systems, we have improved and shown a significant increase in detection sensitivity. The achievements were made by: (a) increased exposure of antibody to the binding region during surface modification, (b) amplification of signal due to GNS and Silver Stain, and (c) the small size of GNS functionalized with PEG-AR conjugation targeting only specific viral particles/antigen, leads to increased antibody-antigen/viral particle binding, and coupled with our ELISA to be sensitive. Future direction for the project includes incorporating into microfluidic system with fiber-optics / LED.

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Guide for Contributors

Aims and Scope

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