Ultralow Detection of Bio-markers using Gold Nanoshells

D. N. Patel*, [1], X. Sun*, G. Zhang*, A. M. Gobin*, [2], R. S. Keynton*,
*University of Louisville, Louisville, KY 40292
dnpate01@gmail.com[1], andre.gobin@louisville.edu[2]

ABSTRACT

The use of Gold nanoparticles (AuNPs) as identification markers or labels, allows 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). 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 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 3 order of magnitude improvement over that previously reported.

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 (SPE), 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 (AuNPs) as identification markers or labels can allow for detection of antigens, analytes, biomarkers, or viral particles, in small sample volumes and low concentrations [1]. By optimizing AuNPs 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 (LOC) as a portable diagnostic tool.

Gold nanoshells (GNS) are spherical, layered, optically tunable spherical nanoparticles, composed of a dielectric core covered by a thin gold shell, with diameters ranging from 10 to 200 nm [8]. GNS have optical, chemical, and physical, which make them an ideal candidate for enhancing cancer detection, cancer treatment, cellular imaging and medical bio sensing.

The geometry of the GNS determines the optical properties. 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 and biocompatibility. 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 [8]. This system has the capability to be utilized in diagnosis of respiratory viruses, cancer, HIV, hepatitis B, C and E, herpes simplex virus, and detection of bioterrorism agents in the modern era [15].

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 [4]. 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 [5, 6]. To overcome these problems, a homogeneous immunoassay was developed that could be used with serum of whole blood directly. This assay is based on a system that can be measured directly in low volumes of whole blood, and would prove greatly advantageous for space flight based diagnostic equipment. Hirsch et al. developed a nanoshell-based immunoassay that overcomes many of the limitations of standard ELISAs. In that work, detection was shown in samples as high as 20% whole blood with detection levels down to 0.8 ng/ml in 30 minutes [5]. 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. In our work, we dramatically improve the sensitivity of this system by careful design of the antibody tethering mechanism to optimize exposure to the antigen. To achieve this, GNS coated with thiol–poly(ethylene) glycol–amine (SH-PEG-NH_2) - Protein G (ProG) conjugate was used to

orient subsequent antibodies for capturing antigens of interest.

Protein G (ProG) is a protein, found on the surface on a variety of staphylococci and streptococci, which has the ability to specifically bind to antibodies at the F_c domain region [9, 10]. Using protein and gene sequence analysis it was reported that ProG is similar to Protein A (ProA) [11]. ProG binds human IgG of all four subclasses and also to mouse monoclonal IgG, with an affinity constant that is higher than of ProA [10]. 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.

The system reported here, uses a modular conjugation assembly based on ProG to create an antibody-ProG-GNS particle. This particle system was exposed to samples of known analyte concentrations ranging from ng/mL to pg/mL levels, while monitoring 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

2.1 GNS Fabrication & Characterization

GNS were fabricated as previously described using [14]. First, silica cores were synthesized using the Stöber method. Next, silica nanoparticles were functionalized with amino groups using 3-Aminopropyl trimethoxysilane (APTES, 99%, Sigma). Gold colloid were prepared to a size of 2-4 nm using the method of Duff et al [17], and aged 3 weeks at 4 °C. Gold colloid was then attached to the silica nanoparticles surface, to act as nucleation sites for the reduction step, and to allow formation of a contiguous shell of gold. GNS spectral properties were evaluated with a Cary 80 BIO UV Vis spectrophotometer. Particle size and zeta potential (charge) measurements were made using Zetasizer (Nano-ZS90, Malvern), and particle morphology was assessed by imaging using STEM (Supra 35VP, Zeiss).

2.2 PEG-ProG Conjugate

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 a NanoSep Centrifuge (OD010C33, VWR) and stored at -20° C for later use.

2.3 GNS-PEG-ProG-Anti-Rb Conjugate

PEG-ProG conjugate were added onto GNS, in a 50mM K_2CO_3 solution (2000:1 molar ratio), 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 molar ratio of 5000:1 and reacted with the same conditions 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 650g for 7 mins. The supernatant was removed immediately and aliquots were re-suspended in 5 mL 50mM K_2CO_3.

2.4 Antibody Quantification on GNS

An ELISA was carried out with a modification of the Lowery-Gobin quantification method to determine the number of antibodies attached onto the surface of the GNS [7,12]. Briefly, GNS-PEG-ProG-Anti-Rb conjugate were washed and re-suspended 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 non-specific binding and washed after to remove any unbound or excess antibody. Final GNS conjugates were then reacted with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate for 15 mins and was stopped with H_2SO_4. Finally, the samples were read using BioTek ELx800 plate reader at 450 nm.

2.5 Detection of Antigen/IgG Complex

GNS-PEG-ProG-Anti-Rb were fabricated as mentioned above and blocked with PEG-SH. The GNS-conjugates were washed and re-suspended 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-conjugates 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 retaining the concentration of GNS at 1 OD. Samples were mixed and spectral scans of each sample were collected every 5 mins for 1 hr.

3. RESULTS AND DISCUSSION

3.1 Characterization of GNS

GNS	Peak Wavelength (nm)	Size (nm)	Zeta (mV)
Bare	814	195.2	-61.6
ProG	816	204.4	-57.8
ProG-Ab	823	239.6	-29.5
ProG-Ab-PEG	818	241.2	-27.2

GNS-PEG-ProG-Anti-Rb was synthesized as described above. The particles from each step were characterized for size and charge. Table above shows GNS parameters after each step on the fabrication process; the size change from 195.2 nm to 241.2 nm with a concomitant reduction in the negative surface charge of due to the addition of ProG, Anti-Rb, and PEG-SH.

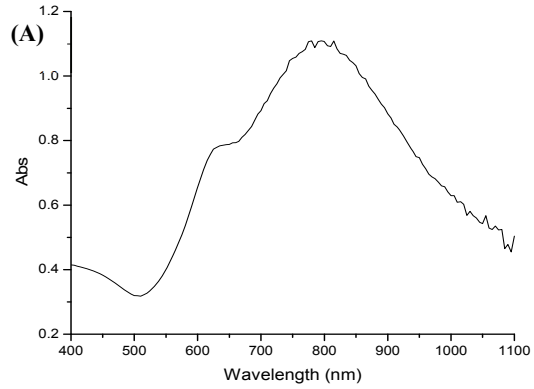

Figure 1: (A) Visible-Near IR spectrum of the as made GNS with peak resonance at 820 nm. (B) Representative STEM image of GNS particles.

3.2 Antibody Loading on GNS

Due to the unique ability of Protein G, to capture multiple antibodies, there was a significant increase in the amount of antibodies bounded on the surface of GNS using this system compared to published data. Prior to running the ELISA, GNS-PEG-ProG-Anti-Rb samples were made using different ratios of antibody to particle, 5000:1, 3000:1, and 1000:1. Using the 5000:1 ratio resulted in approximately 311 antibodies per GNS, Figure 2. The antibody density reported here is double that reported previously [7,12].

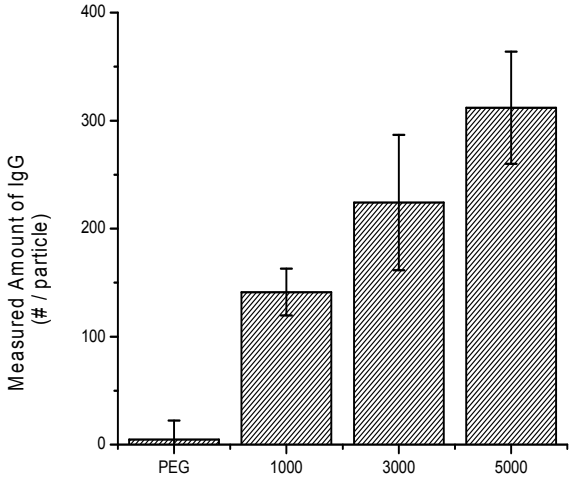

Figure 2: ELISA-based Lowery-Gobin quantification method to determine the number of antibodies on GNS with different antibody: GNS ratios.

3.3 Antigen Detection

Initial studies were focused on analyte detection in saline solutions, using GNS-PEG-ProG-Anti-Rb, which has the capability to quantitatively detect analytes at various concentrations within 10 minutes of mixing.

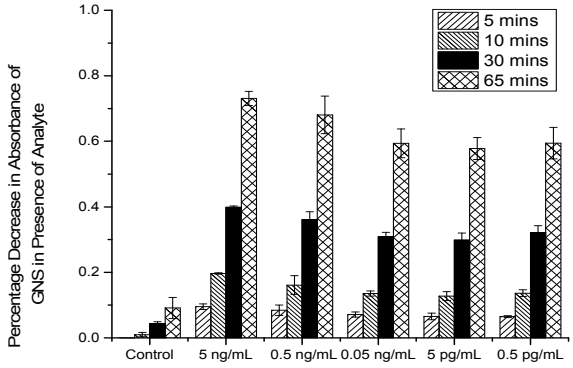

Figure 1: Indicates the percent decrease in the peak spectral absorbance of GNS, from t=0 to 65 minutes, in the presence of analytes, compared to analyte-free sample.

To assess the assay performance, spectral scans for five different analyte concentrations (5 ng/mL to 0.5 pg/mL) in

PBS were performed immediately after mixing conjugated nanoparticles with analyte solutions. The aggregation progression was monitored by changes in the spectra at 820 nm (the designed peak for these nanoparticles). When two GNS antibody conjugates are mixed in with the analytes, this will induce GNS to form dimeric or high-order oligomeric aggregates through an antibody-antigen sandwich.

Due to this phenomenon, the absorbance of the particles decreases over time**.** With increasing concentration of analytes, the assay demonstrated rapid aggregation at early times. All five analytes concentrations were statistically different compared to control samples after 10 minutes. With this system, we can conclude that this immunoassay promises a new standard of detections of analytes, which is fast and accurate at concentrations a thousand fold lower than previously reported systems [5].

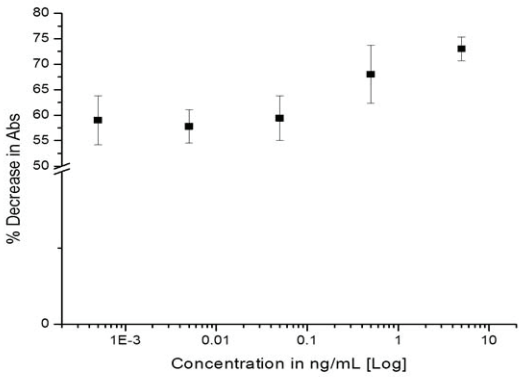

Figure 4: Percentage decrease in absorbance of GNS after 1 hr.

4. CONCLUSION

As evidenced in Figures 3 & 4, we have demonstrated an improvement in detection of analytes using a nanoshell based immuno assay to levels down to 0.5 pg/mL, compared to 0.88 ng/mL, in PBS solutions previously reported, this represents a 1000X increase in sensitivity. The sensitivity increase is attributed to the increased loading and proper orientation of antibodies on the nanoshell. This was accomplished through the use of a PEGylated Protein G on the surface of the nanoshell that binds to the Fc portion of antibodies. Further work is ongoing to evaluate the system in serum and in diluted blood to establish if this increase in sensitivity could be maintained by this system. The ability to use Protein G on the surface allows for a more versatile platform where antibodies against a wide range of analytes can be allowing quicker readiness for detection of agents for which mouse antibodies have been developed. This method also simplifies the chemistry and the stability of the nanoparticles, allowing for longer storage and transport, which in turn increases ability to react to situations requiring rapid detection of certain agents.

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REFERENCES

1. Liu, J. and Y. Lu. Nat Protoc, 2006. 1(1): p. 246-52.
2. Redon, C.E., et al., Adv Space Res, 2009. 43(8): p. 1171-1178.
3. Durante, M., 2005 164(4): p. 467-73.
4. Chen, J., C. J Biomed Opt, 2009. 14(4): p. 040501.
5. Hirsch, L.R., et al., Anal Chem, 2003. 75(10): p. 2377-81.
6. Liu, X., et al., J Am Chem Soc, 2008. 130(9): p. 2780-2.
7. Lowery, A.R., et al., Int J Nanomedicine, 2006. 1(2): p. 149-54.
8. Erickson, T.A., Tunnell, J.W. , Nanomaterials for the Life Sciences Vol. 3: Mixed Metal Nanomaterials.
9. Sun, X.H.Y., D. Q.; J. Membr. Sci. 2009, 344 (1-2), 165-171.
10. Guss, B., et al., EMBO J, 1986. 5(7): p. 1567-75.
11. Goward, C.R., et al., *G.* Biochem J, 1990. 267(1): p. 171-7.
12. Gobin, A.M., et al., Nano Lett, 2007. 7(7): p. 1929-34.
13. Gobin, A.M., et al., Lasers in Surgery and Medicine, 2005. 37(2): p. 123-129.
14. Oldenburg, S.J., et al., App. Phys. Lett., 1999. 75(19): p. 2897-2899.
15. Abraham, A. M., R. Kannangai, et al. (2008). Indian journal of medical microbiology 26(4): 297-301.