31st Annual International Conference of the IEEE EMBS
Minneapolis, Minnesota, USA, September 2-6, 2009

Multi-Color Raman Nanotags for Tumor Cell Biomarker Detection

James Nyagilo, Ming Xiao, Xiankai Sun, Digant P. Davé

Abstract—**We report the synthesis and characterization of multi-color gold nanoparticle based tags (Raman Nanotags) for molecular imaging. The multi-color Raman Nanotags are PEGylated gold nanoparticles (AuNPs) encapsulating a Raman reporter dyes which can be functionalized with any ligand of interest for targeted molecular imaging. The Raman Nanotags synthesized with 65 nm gold nanoparticles exhibit the largest surface enhanced Raman scattering (SERS) signal. Results are presented quantify the measured SERS signal, dynamic range, reproducibility, and stability of Raman Nanotags. In vitro cell culture experiments for targeted biomarker detection using functionalized Raman Nanotags are also presented.**

I. INTRODUCTION

Metal nanoparticles exhibit unique optical properties due to their size that is not exhibited by their bulk counterpart. These properties can be exploited for biomedical imaging and sensing application to improve contrast and enhance sensitivity. Functionalizing metal nanoparticles permits targeted biomolecule specific detection. AuNPs are of particular interest since they are considered less cytotoxic compared to fluorescent dyes, quantum dots and carbon nanotubes and gold provides an excellent surface for functionalization using the thiol chemistry. These advantages make AuNPs a robust platform for developing functionalized contrast agent to be used for diagnostic molecular imaging applications.

Raman spectroscopy is one of the most sensitive techniques for chemical analysis given the unique spectral fingerprint of every chemical compound. A major weakness of Raman spectroscopy is the poor efficiency of Raman scattering which has limited its use in bioimaging and biosensing. Efficiency of Raman scattering can dramatically increase (enhancement factor - 10^6 to 10^{14}) when a molecule of interest is in close proximity to a nanosurface [1,2]. This enhancement is possible due to a phenomenon known as Surface Enhanced Raman Scattering (SERS). With such large enhancement of Raman scattering the use of SERS for biomolecular sensing is an attractive alternative to fluorescence. Fluorescent tags have emerged as dominant optical contrast agents for molecular imaging for a wide variety of in vitro applications. *In vivo* applications of organic fluorescent dyes have been limited due to auto-fluorescence of tissue and their cyto-toxcitiy. Fluorescence tags suffer from photo-bleaching and only limited tags (two or three) can be simultaneously detected due to spectral overlap. These limitations can be addressed by using SERS to achieve detection sensitivity that potentially exceeds that of fluorescence techniques and also achieve multiplexed detection of biomolecules.

Despite unique advantages offered by SERS, lack of signal reproducibility and quantification has prevented their wide spread use for *in vitro* or *in vivo* imaging and sensing applications until now. The lack of signal reproducibility can occur due to variation in NP size and distribution or aggregation. Metal nanoparticles tend to aggregate in commonly used buffers and serum. Although aggregation does enhances the SERS signal but from a practical standpoint it is undesirable since aggregation cannot be controlled, resultant SERS fluctuates, and its substantially size is of little use for any *in vivo* applications. In the last couple of years a very promising strategy to synthesize Raman Nanotags [2,4] for *in vitro* and *in vivo* application has emerged. The strategy involves encapsulating organic dyes as signature reporter dye between AuNP and a layer of silica or PEG. Target specific ligand can be attached to PEG or silica surface with well established bioconjugation chemistries.

We have systematically synthesized Raman Nanotags of sizes ranging from 50 to 120 nm to determine the optimum size for SERS. Raman Nanotags of five different colors have been synthesized and characterized. Functionalized Raman Nanotags for targeted detection of biomarker were tested in tumor cell expressing/over expressing epidermal growth factor repector (EGFR). We also synthesized Raman nanotags with commercially available AuNPs (60 nm, TedPella) for comparison.

II.METHOD AND PROCEDURE

We have been able to reproducibly synthesize AuNPs with five distinct sizes in the range of 50 – 120 nm (Fig. 1). A smaller AuNP (16 .7 ± 1.7 nm) is prepared first and then used as seeds to synthesize the larger AuNPs. The AuNPs were characterized by UV-Vis Spectrocopy, Transimission Electron Microscopy (TEM), and Dynamic Light Scattering (DLS).

The gold nanoparticles (AuNPs) were synthesized using the Fren's Method [5] and a seed growing method described by Jiali Niu [6]. An aqueous solution containing 50 mL of 2.5 $\times 10^{-4}$ M $HAuCl_4$ was heated to a boil with constant stirring, to which 1.75 mL of 1% (wt) aqueous sodium citrate was

Manuscript submitted on April 23, 2009. This work was supported in part by the U.S. Department of Energy and National Institutes of Health

J. Nyagilo and D.P. Davé are with the Bioengineering Department at The University of Texas at Arlington, Arlington, TX 76019, USA

M. Xiao and X. Sun are with the Radiology Department at University of Texas Southwestern Medical Center, Dallas TX 75203, USA

added. Within a minute, the solution turned from faint blue to red indicating the formation of AuNPs. The boiling and stirring were continued for 30 min. The resulting solution was then cooled down to room temperature, which afforded AuNPs with a mean diameter of 16.7 ± 1.7 nm as determined by TEM. The concentration of the AuNPs was estimated to be 1.1×10^{15} AuNPs/L. Gold nanoparticle with different sizes were prepared by adding different volumes of the solution of the small AuNPs (16.7 ± 1.7 nm) to each of four 125 mL conical flasks containing 240 µL of 0.01 M MSA solution and 160 µL of 2.5×10^{-2} M $HAuCl_4$ (maintaining a constant ratio of [$HAuCl_4$]:[MSA]) under rigorous stirring.

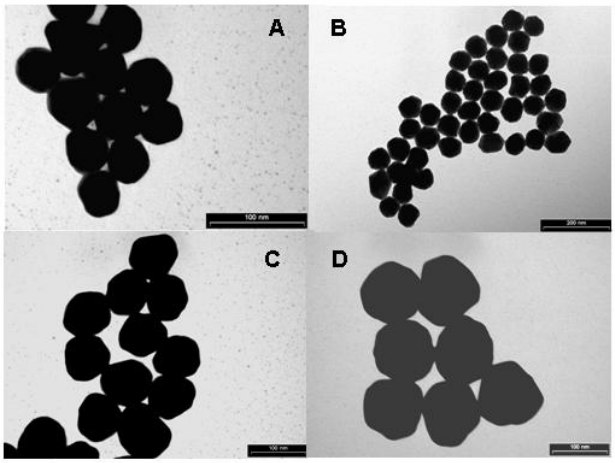

Figure 1. TEM images of gold nanoparticles (A) 51 ± 3.1 nm, (B) 65 ± 3.6 nm, (C) 83 ± 5.1 nm and (D) 114 ± 7.9 nm. The scale bars for (A), (B) and (D) read 100 nm while that of (B) is 200 nm

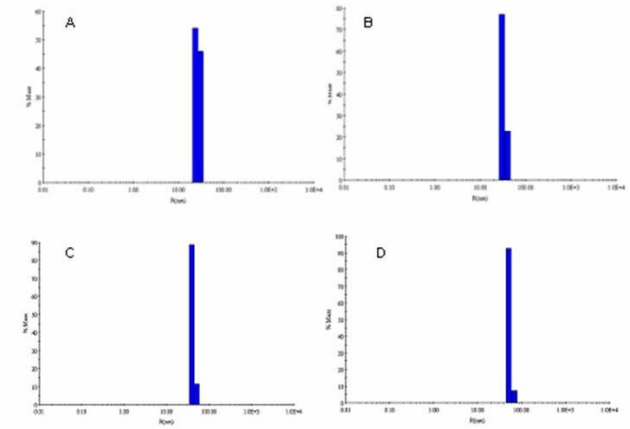

Figure 2. A) – (D) Dynamic light scattering (DLS) histograms of the gold nanoparticles (Fig.1) respectively.

Synthesis of the nanotags involves encoding gold nanoparticles with a Raman reporter dye followed by a coating of thiolated polyethylene glycol (mPEG-SH: the end of PEG is methyl capped) and thiol modified heterofunctional polyethylene glycol (HS-PEG-COOH). Fig. 3 illustrates the process of Raman Nanotags synthesis. The process in described in detail in ref. 3. Using this process Raman Nanotags of five distinct colors (reporter dyes) were synthesized.,

To carry out functionalization, carboxylate groups on the surface of Raman Nanotags were first reacted with EDC (*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) and sulfo-NHS (*N*-Hydroxysulfosuccinimide) to form an activated ester, which was then reacted with the primary amine groups on biomarker ligand of interest. The resulting ligand-Raman Nanotag conjugates were further purified by either size-exclusion column separation or centrifugation.

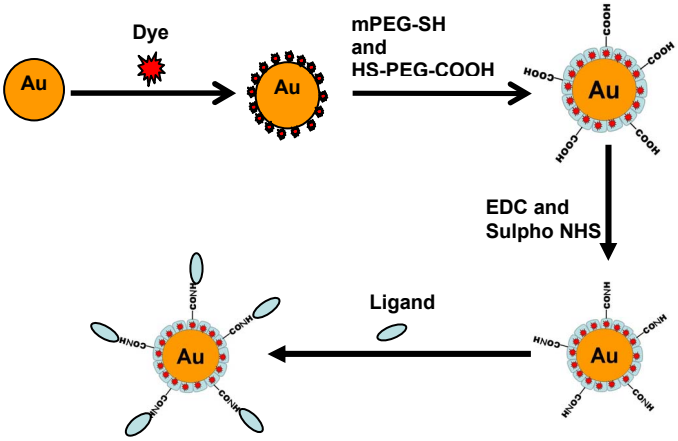

Figure 3: Schematic of the process of making the Raman Nanotags including encoding the nanoparticles, PEGlyation, activation and functionalization.

III. RESULTS AND DISCUSSION

SERS signals of synthesized multi-color Raman Nanotags were measured with a home built Raman spectroscopy module. The module was designed to be integrated into the side port of an inverted microscope for simultaneous microscopy (DIC and fluorescence) and Raman spectroscopy. Excitation laser wavelength was 785 nm. All the presented spectra were collected by a 40X microscope objective and recorded by a high resolution spectrometer (Ocean Optics QE65000). Recorded SERS signals of various color Raman Nanotags is shown Fig. 4

As opposed to AuNPs the synthesized Raman Nanotags remain stable under a variety of harsh conditions (Fig. 4). Raman Nanotags were tested for stability by centrifugation and resuspension in various solvents or media, such as methanol, DMSO, MES, PBS, or goat serum.

SERS signal of Raman Nanotags of 65.7 nm size was the largest of the five sizes of AuNPs that were synthesized and 60 nm commercially purchased AuNPs (Fig. 6a&b). It is noteworthy that the 65.7 nm sized AuNPs are quasispherical which likely results in the greater SERS signal than other spherical AuNPs (e.g. the commercial available ones).

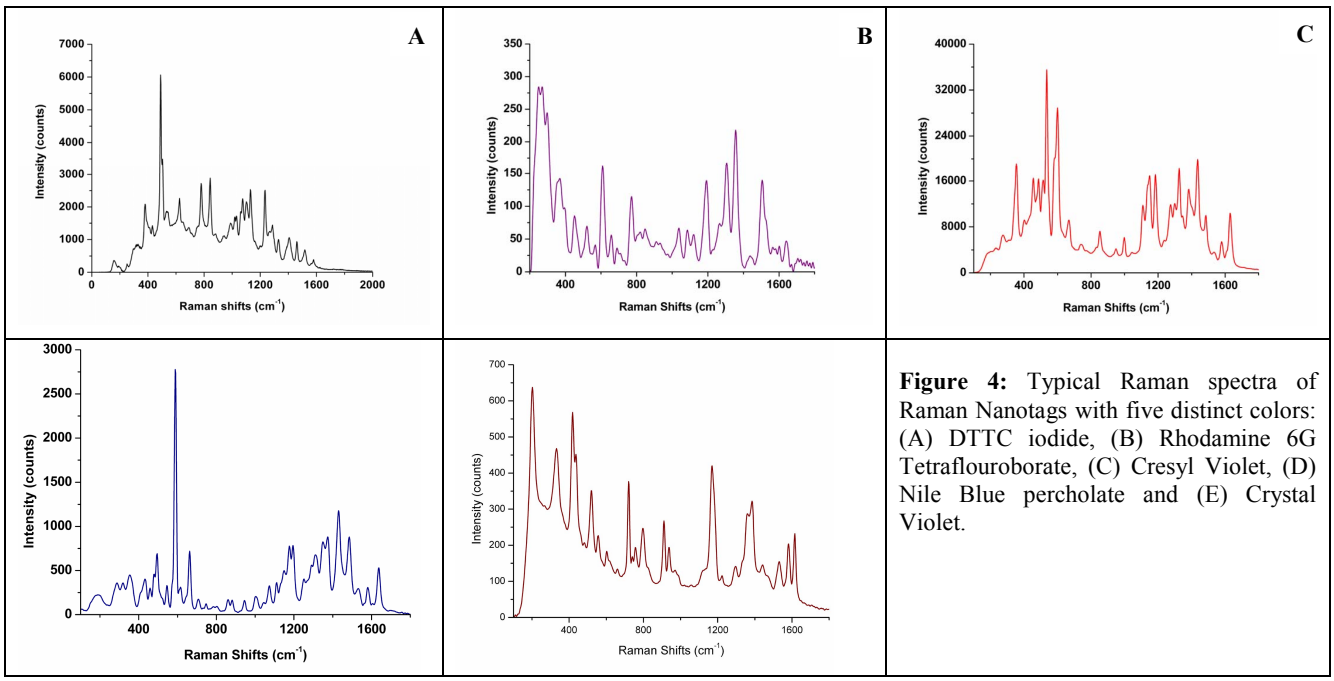

Figure 4: Typical Raman spectra of Raman Nanotags with five distinct colors: (A) DTTC iodide, (B) Rhodamine 6G Tetraflouroborate, (C) Cresyl Violet, (D) Nile Blue percholate and (E) Crystal Violet.

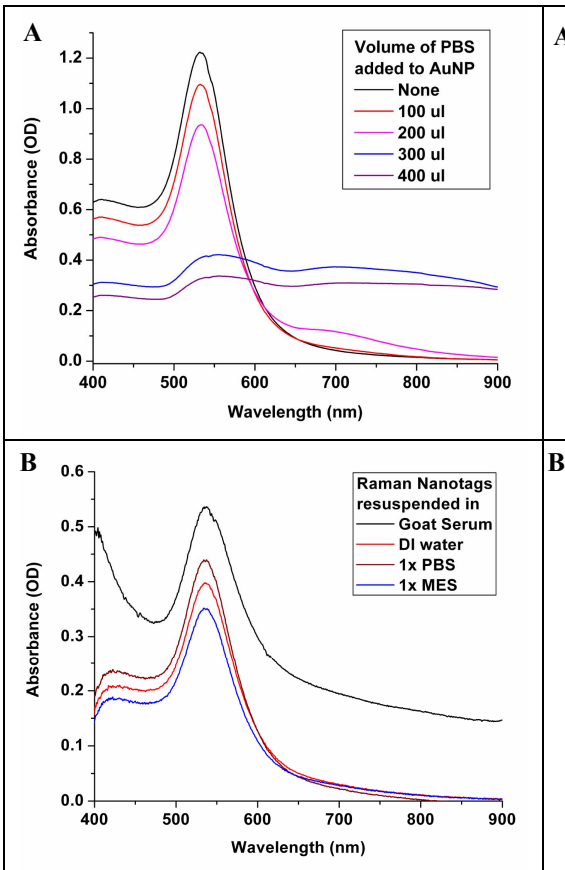

Figure 5. Effect of "harsh" solvents or media on (A) AuNPs and (B) Raman Nanotags.

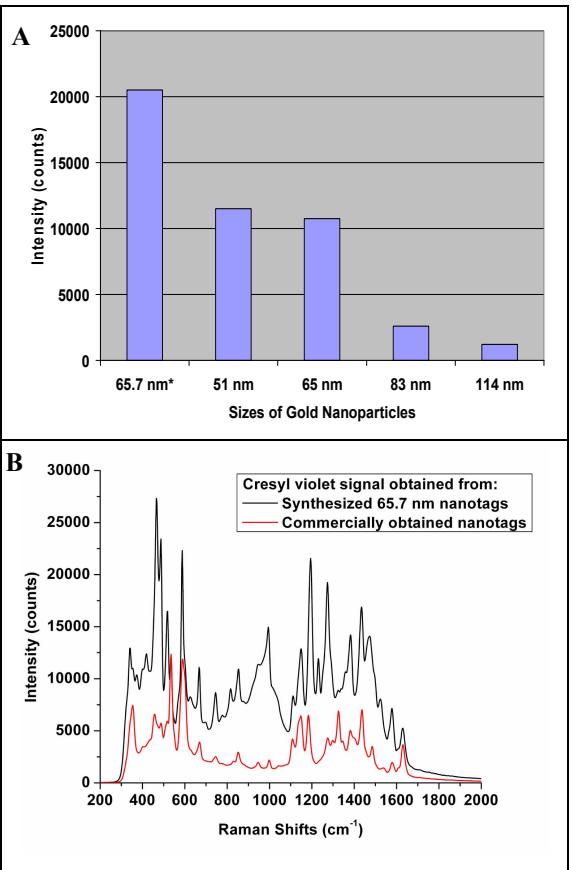

Figure 6. (A) SERS intensity at 587.84 cm^{-1} compared for 5 Raman Nanotags made using AuNPs of different sizes. (B) Comparison of SERS spectra Raman Nanotags made from 65.7 nm AuNPs and commercially obtained 60 nm AuNPs. *The 65.7 nm Nanoparticles are made using Fren's Method.

The SERS signal from Raman Nanotags have linear detection dynamic range (Fig. 7) of about 30 db from 10^{11} to 10^{14} particles per liter (1.83 pmol to 18.3 nmol).

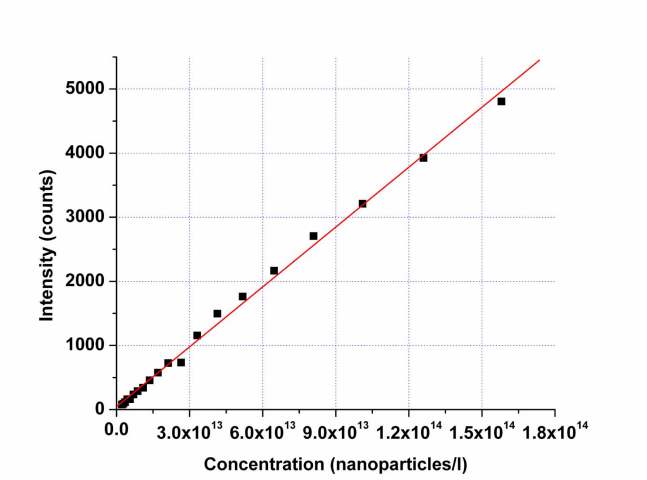

Figure 7: Measured dynamic range of 65.7 nm Raman Nanotags.

In vitro cell culture experiments were performed using U87MG (Human glioblastoma-astocytoma) cell lines and U87MG.EGFr cell lines. U87MG cell line express EGFR wheras U87MG.EGFr over express EGFR. Raman Nanotags functionalized with monoclonal mouse anti-human EGFR (Dako Inc.) were incubated in cell cultures after fixation for period of 12 hrs at 4^0 C. After gently washing the unattached Raman Nanotags (3X PBS) goat anti-mouse IgG Alexa Fluor 594 was incubated in cell culture for a period of 4 hrs at room temperature and then washed (3X PBS).

DIC, SERS signal and fluorescence images were obtained of the samples (Fig. 8). Fluorescence images (Fig.8B) confirm that immunoreactivity of the EGFr antibody remains active after conjugation to Raman Nanotags, and show signals obtained from U87MG cells expressing higher levels of EGFr have higher SERS signal intensities (Fig. 8D) compared the ones expressing low levels of EGFr (Fig. 8F). Blank areas show no SERS signal (Fig. 8H) from the Raman Nanotags. The signal seen is a background signal from the glass coverslip.

In conclusion we have synthesized and characterized multi-color Raman Nanotags of various sizes. Raman Nanotags synthesized with 65 nm AuNPS exhibit the largest SERS signals. The synthesized Raman Nanotags can be potentially used for quantitative multiplexed molecular profiling of multiple tumor cell biomarkers in an *in vitro* or *in vivo* setting.

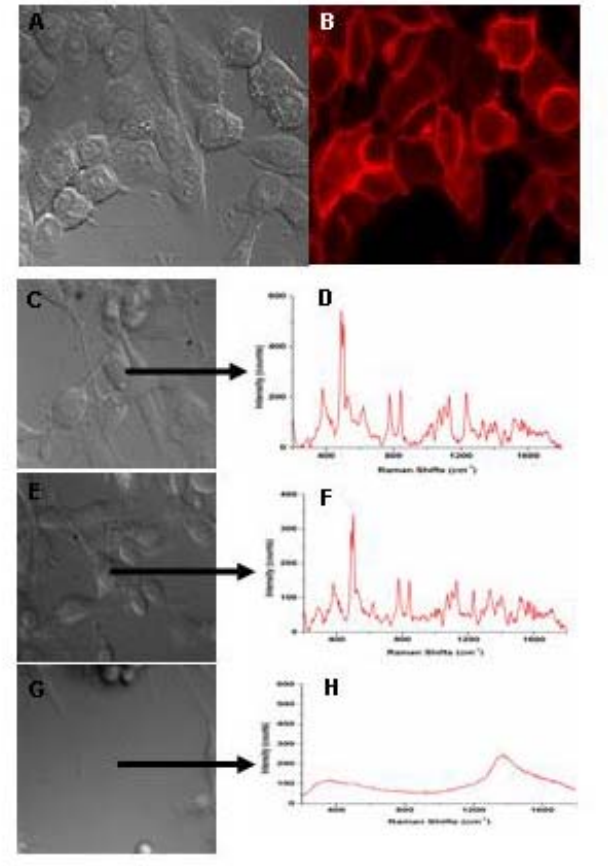

Figure 8: (A) DIC and (B) fluorescence images of U87MG.EGFr cell lines conjugated with Raman Nanotags. (C)-(H) DIC images and SERS spectra obtained from U87MG cells, U87MG.EGFr cells, and a blank area respectively.

VI. REFERENCES

1. K. Kneipp, Y. Wang, H. Kneipp, L. T. Perelman, I. Itzkan, R. R. Dasari, and M. S. Feld. "Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS)". Phys. Rev. Lett. 78, 1667-1670 (1997).
2. Shuming Nie, Steven R. Emory. **"**Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering". Science 275 1102 – 1106 (1997).
3. X. Qian, X.-H. Peng, D. O. Ansari, Q. Yin-Goen, G. Z. Chen, D. M. Shin, L. Yang, A. N. Young, M. D. Wang, and S. Nie. "In vivo tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags". Nature Biotechnology 26 83-90 (2008).
4. Jong-Ho Kim, Jun-Sung Kim, Heejeong Choi, Sang-Myung Lee, Bong-Hyun Jun, Kyeong-Nam Yu, Eunye Kuk, Yong-Kweon Kim, Dae Hong Jeong, Myung-Haing Cho, Yoon-Sik Lee. "Nanoparticle Probes with Surface Enhanced Raman Spectroscopic Tags for Cellular Cancer Targetin"g. *Anal. Chem.* 78 6967–6973 (2006).
5. Frens, G. "Controlled nucleation for the regulation of the particle size in mono disperse gold suspensions". *Nature: Phys. Sci*. 241 20-22 (1973).
6. Niu J, Zhu T, Liu Z. "One-step seed-mediated growth of 30–150 nm quasispherical gold nanoparticles with 2-mercaptosuccinic acid as a new reducing agent". *Nanotechnology* 18 325607 (2007).