Preparation of Composite Organic-Inorganic Nanoparticles (COIN) with Distinctive Raman Signatures

Jingwu Zhang, Yu Lu, Manqiu Cao, Haoyu Qin, Mineo Yamakawa, Claire Dentinger, Lienchi Nguyen, AJ Chmura, Jing Zhu, Barry Lutz, Kenneth Swartz, Liming Wang, Xing Su, and Lei Sun

Biomedical/Life Sciences, Digital Health Group, Intel Corporation, Santa Clara, CA, USA

ABSTRACT

Composite organic-inorganic nanoparticle (COIN) is a new type of optical label for multiplex biomolecule detection. It has strong Raman signal intensity and the potential for a large number of unique Raman signatures. We have developed methods to fabricate, encapsulate, and to functionalize COINs with antibodies. We have demonstrated that COINs can give comparable detection limit and linearity range to "golden standard" protein detection method – ELISA, but without secondary antibody binding and enzyme amplification.

Keywords: SERS, nanoparticles, Raman, immunoassay, tissue analysis

1 INTRODUCTION

Various types of nanoparticles with unique optical properties have been developed in recent years and successfully used as tags in biological assays. For example, quantum dots have been shown to be able to replace the fluorescence dyes in bio-tagging applications with the added advantages such as greater signal intensity, narrower emission peak, longer fluorescence time and photostability [1,2]. Moreover, as their emission wavelength is size dependent, several colors of quantum dots in the visible light range are available, making it possible to use them in multiplex assays. A different method to achieve greater photostability and emission intensity is by doping silica nanoparticles with traditional fluorescent dyes [3]. Those dye doped silica particles are easy to solublize and functionize with different biomolecules. However, the broader emission spectra of the traditional dyes are likely to be a limiting factor for multiplex assay development in solution.

Another novel type of tagging nanoparticle which emerged in the last few years is based on Surface-Enhanced Raman Scattering (SERS) [4-9]. Although typical Raman scattering efficiency is extremely low compared to fluorescence emission of fluorophores, the Raman signal can be greatly enhanced if the molecules are adsorbed onto the surface of metal nanoparticles or roughened metal substrates. This SERS phenomenon can provide many orders of magnitude of enhancement in Raman signal and the detection of single molecules has been reported using SERS [10, 11]. SERS has several advantages over fluorescence-based detection systems, such as: (i) no photobleaching, (ii) potential for higher multiplexing with minimal spectral overlap due to narrower Raman peak-widths, (iii) single laser excitation for multiple SERS-based tags, and (iv) increased number of SERS-based tags with distinctive spectral features.

SERS-based tags have been developed by several groups using single metal nanoparticles with Raman active molecules on the metal surface [5-7]. The tag nanoparticles can be further coated with a thin layer of silica to make the particles more stable and easier to functionize. However, the SERS effect is not maximized as only single metal particles rather than their aggregates are involved. It is now well-known that the molecules situated at metal particle junctions can produce Raman scattering intensity several orders of magnitude higher than the same molecule on the surface of single particles. At Intel, we have developed a new type of Raman tag called composite organic-inorganic nanoparticles (COIN) [8, 9]. Unlike Raman tags based on individual metal particles, COINs are clusters formed from metal nanoparticles in the presence of organic Raman active molecules (Raman labels). We have developed a simple and highly scalable method to incorporate a variety of Raman labels into COINs. We have also developed procedures to encapsulate COINs by cross-linking biopolymers with functional groups for conjugation with different antibodies. In this report, we present the methods that we developed for COIN synthesis, encapsulation and functionization. We also present the results of using the COIN in a sandwich assay for protein detection.

2 PREPARATION

The preparation of COIN suitable for bioassays involved multiple steps as shown in Figure 1. First, small silver seed nanoparticles (<12nm) are prepared. The seed nanoparticles are mixed with Raman labels to produce clusters by aggregation while the seed particles grow through silver deposition. Once the cluster reaches a desired size, aggregation is stopped by addition of bovine serum albumin (BSA). Seed particles and very small clusters are then removed by centrifugation during enrichment. After that, additional BSA molecules are added and COINs are encapsulated by cross-linking BSA molecules to provide additional colloidal stability and active groups for functionization with antibodies. Details are provided for each step in this section:

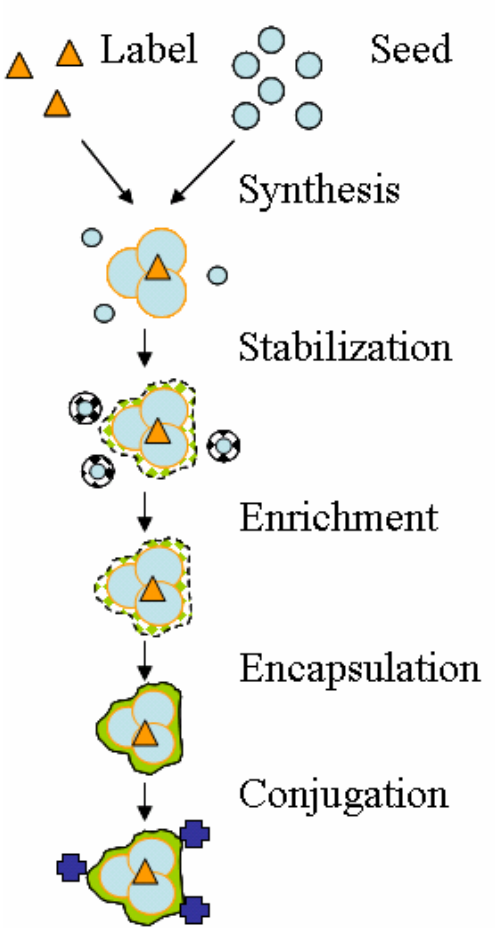

Figure 1: Flow chart for COIN reagent preparation

2.1 Seed Crystal Preparation

Silver seed particles are prepared by reduction of silver nitrate with sodium borohydride at room temperature. 50ml of solution A containing 0.6mM Sodium Borohydride (from 50mM stock solution prepared within an hour), 2mM NaOH and 8mM sodium citrate is rapidly added to 50ml of solution B containing 4mM silver nitrate in a 125mL glass bottle under agitation with a magnetic stirrer. The bottle is shaken by hand vigorously for 5 seconds to ensure rapid mixing. The particle size is analyzed by photon correlation spectroscopy (PCS, Nano-ZS, Malvern). Typically the z-average diameter is 11±1nm with a polydispersity index of <0.2. It is interesting to note that adding solution B into solution A lead to particles with greater polydispersity. The silver suspension should be stored in the dark. While sodium hydroxide is added to neutralize released protons during the reaction, sodium citrate is used as a stabilizing agent. Without citrate ions, significant aggregation occurs within 24 hours. The addition of citrate extends the colloidal stability to about one week. However, the particle size is verified by PCS measurement to ensure that there is no significant aggregation before use.

2.2 COIN synthesis and size enrichment

COINs can be prepared conveniently by using a convection oven. Silver seed suspension is mixed with sodium citrate and silver nitrate solutions in a 20 mL glass vial. The final volume of the mixture is typically 15 mL, which contained silver particles (equivalent to 0.5 mM Ag^+), 1.0 mM silver nitrate and 2.0 mM sodium citrate (including the portion from the seed suspension). A range of label concentrations are added to the above solution to determine the optimum concentration. The glass vials are heated in the oven at 95 °C for 30-60 min. During heating, the seed particles grow due to silver deposition and, at the same time, aggregation takes place as the adsorption of Raman labels on silver surface decreases the electrostatic repulsion between silver particles. After heating for a controlled period of time, the suspensions are taken out of the oven and cooled at room temperature. PCS is used to monitor the size changes of COINs to determine the optimum reaction time. 0.1-0.5% BSA is added as soon as the clusters reach the desired size (50-80nm).

The Raman label concentration is a critical parameter for COIN synthesis. The optimum label concentration is typically determined empirically. If the concentration is too low, there will be no significant cluster formation and the resulting suspension will have very low Raman scattering intensity. In contrast, when the label concentration is too high, rapid aggregation between particles will occur, leading to severe sedimentation. Once the optimum label concentration has been determined, a COIN sample can be reproducibly prepared: typically a standard deviation of less than 15% could be achieved in both Raman signal intensity and average COIN size.

As COINs are nano-clusters formed by random aggregation, the sample contains single particles and very small clusters. Thus differential centrifugation is used to remove the small particles which have relatively low Raman signal intensity. Typically, 25ml of COIN suspension contained in 50ml conical tube is centrifuged in a swing bucket rotor at 4500g for 15min (Eppendorf 5804 with the A-4-44 Rotor). After discarding the supernatant, the pellet is resuspended in 1mM sodium citrate containing 0.5% BSA. Figure 2 shows the transmission electron micrograph of COINs after size enrichment.

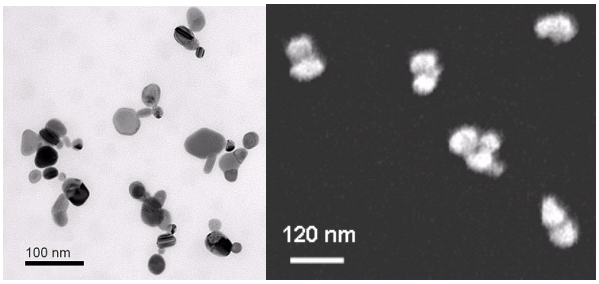

Figure 2: Transmission and scanning electron micrographs of COINs after size enrichment

NSTI-Nanotech 2007, www.nsti.org, ISBN 1420061836 Vol. 2, 2007

2.3 Encapsulation

Encapsulation is a crucial step before nanoparticle tags can be applied in a bioassay. It stabilizes the nanoparticles by preventing aggregation and introduces surface functional groups for biomolecule attachment. During COIN preparation and enrichment, BSA is used to stabilize the COIN suspension. As the BSA molecules are physically adsorbed on COIN particles, desorption may occur under different assay conditions. Thus, we developed an encapsulation procedure by cross-linking the adsorbed BSA macromolecules with glutaraldehyde.

COIN suspension is adjusted to about $2.5x10^{12}$ particles per mL (~ 4 nM particle concentration) with 1mM sodium citrate containing 0.5% BSA. 35% glutaraldehyde is added to the COIN suspension at a volume ratio of 1 to 17. The cross-linking reaction is allowed to proceed for 4 hours at room temperature. The mixture is then centrifuged to remove the excess aldehyde groups, and the pellet is re-suspended in a buffer solution containing 10mM glycine and 10mM sodium citrate (pH8). After 30 minutes, the suspension is centrifuged, and the supernatant is discarded. The pellet is washed twice with, and re-suspended in, 1mM sodium citrate solution. It is necessary to use excessive amounts of BSA to have complete encapsulation. Thus, it is probable that BSA macromolecules are cross-linked in solution before reacting with BSA molecules adsorbed on COIN surface.

Raman signal interference from cross-linked BSA is negligible as compared with the high intensity of signals from the Raman label molecules in COINs. A 20%-50% decrease in Raman signal intensity is often observed after the encapsulation process, which may be caused by loss of Raman active molecules previously adsorbed on the COIN surface (see Fig. 3) We monitored the size and Raman intensity of encapsulated COINs for over 6-months while stored at 4°C. Data showed that the encapsulated COINs were stable during that period of time.

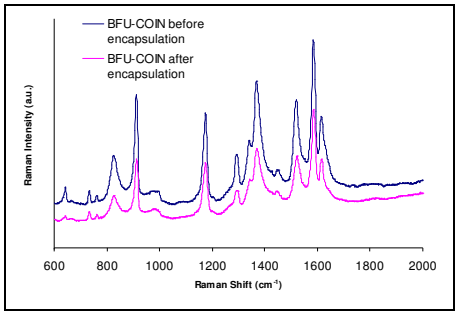

Figure 3. Raman spectra of BFU-COIN before and after encapsulation

2.4 Conjugation

The encapsulated COINs have a large number of carboxylic acid groups from the cross-linked BSA macromolecules on the particle surface, which can be conjugated to free amine groups of various antibodies. For instance, COIN-Anti-IL8 conjugates may be prepared as follows: Encapsulated COINs are centrifuged and re-suspended in 10mM borate buffer at pH 7.5. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) is added in excess and allowed to react for 5 minutes. The excess amount of EDC is subsequently removed by centrifugation. An approximately 1000-fold excess of the anti-IL8 antibody is added to react with the activated COIN surface for 40 minutes. After that, the COINs are washed 3 times to remove the free antibody, and re-suspended in 10 mM borate solution with 1% BSA and 0.05% Tween-20.

3 APPLICATION

Thanks to their high signal intensity, individual COIN particles are detectable by Raman spectroscopy. To demonstrate this, 10µl of COIN suspension was spread out within a volume of $10x10mm^2$ area with ~100µm height between two glass slides. The COIN suspension was diluted down to near single particle per scanning frame of a laser detection volume. The results indicated COINs could be detected at single particle level (Fig. 4).

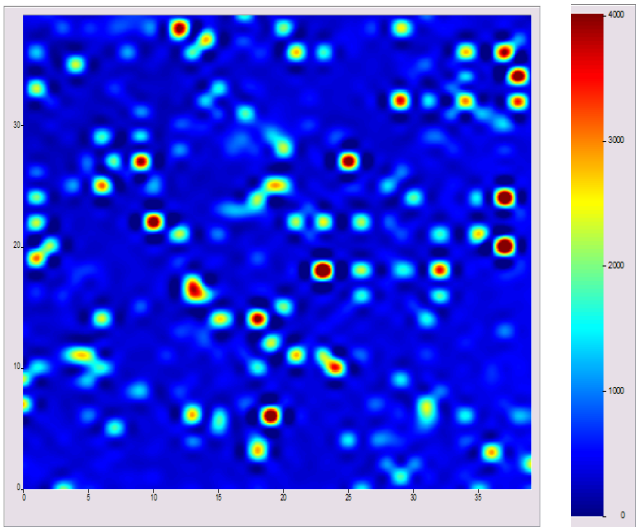

Figure 4: Raman intensity map of diluted COIN suspension in a thin chamber.

We used an assay scheme similar to a sandwich immuno assay to show that COINs can be used as Raman tags for bio-analyte detection. To determine the detection sensitivity, we applied interleukin-8 protein (IL8, 0.1 to 1000 pg/mL) to a Xenobind™ Aldehyde slide (Xenopore Inc., NJ, USA) coated with anti-IL-8 capture antibody. Then COIN-anti-IL8 conjugates were applied. After 30 minutes of incubation, unbound COIN conjugates were washed away. The substrate was scanned under Raman spectroscopy and the Raman spectra were recorded. The laser power at the sample was ~60 mW and the excitation wavelength was 514 nm.

The laser beam was about 4 microns in diameter. Spectra were collected from one sample by continuously moving the motorized stage; each spectrum represented the information collected over 100 milliseconds. The average Raman intensity at a selected wavelength is plotted against analyte concentration in Figure 5. As can be seen, a detection limit of 1pg/mL can be achieved, which is comparable to that of ELISA. The linear range of detection for both methods is also comparable. However, with COINs, the assay procedure is significantly simplified by eliminating enzyme amplification.

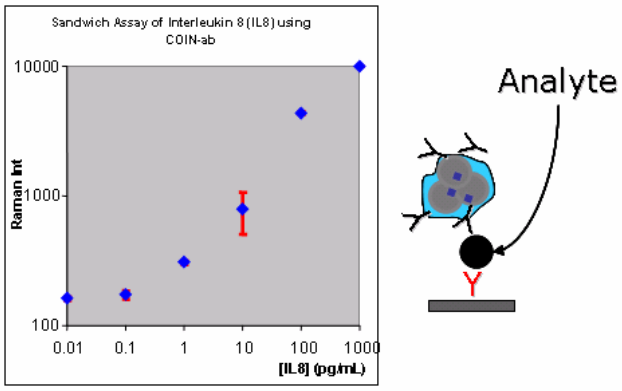

Figure 5: Detection of Interleukin 8 by Sandwich assay using COIN-antibody conjugates.

4 SUMMARY

We have developed a general and scalable method to prepare SERS-based tag nanoparticles. Our fabrication procedure takes the advantage of the fact that the surface enhancement factor is greatest when the molecules are situated at the junctions of noble metal nanoparticles. Highly Raman active organic compounds (Raman labels) are selected and used to induce the cluster formation of silver nanoparticles. Various types of organic compounds can be incorporated into the nanoclusters to produce COINs with distinctive Raman signatures. The aggregation kinetics and cluster size are controlled by the Raman label concentration, temperature and aggregation time. Once the clusters reach a desired size, a stabilizing agent such as bovine serum albumin (BSA) is added to stop further aggregation. The nanoparticles are subsequently encapsulated to enhance their stability; and then functionalized by biomolecules such as antibodies for application in biomolecule detection. We have show that COINs can be used in a sandwich-assay and produces comparable results in terms of detection limit and dynamic range as ELISA. However, the high signal intensity of the tag nanoparticles eliminates the need for the secondary antibody binding and enzyme amplification.

REFERENCES

[1] M. Bruchez, M. Moronne, P. Gin, S. Weiss and A.P. Alivisatos, Science 281, 2013-2016, 1998.

[2] W.C.W. Chan and S.M. Wang, Science 281, 2016-2018, 1998.

[3] G. Yao, L. Wang, Y. Wu, J. Smith, J. Xu, W. Zhao, E. Lee and W. Tan, Anal. Bioanal. Chem, 385, 518-524, 2006.

[4] Y. Cao, R. Jin, C. Mirkin, Science 297 1536-1540, 2002.

[5] S.P. Mulvaney, M.D. Musick, C.D. Keating and M.J. Natan, Langmuir 19, 4784-4790, 2003.

[6] W.E. Doering, S. Nie, Anal. Chem, 75, 6171-6176, 2003.

[7] D.S. Grubisha, R.J. Lipert, H.Y. Park, J. Driskell, M.D. Porter, Anal Chem. 75, 5936-5943, 2003.

[8] X. Su, J. Zhang, L. Sun, T.-W. Koo, S. Chan, N. Sundararajan, M. Yamakawa and A.A. Berlin, Nano Lett, 5, 49-54, 2005

[9] L. Sun, K.-B. Sung, C. Dentinger, B. Lutz, L. Nguyen, J. Zhang, H. Qin, M. Yamakawa, M. Cao, Y. Lu, A. Chmura, J. Zhu, X, Su, A.A. Berlin, S. Chan, and B. Knudsen, *Nano Lett.* 7, 351-356, 2007.

[10] J. Jiang, K. Bosnick, M. Maillard and L. Brus, J. Phys. Chem. B, 107, 9964-9972, 2003.

[11] T.W. Koo, S. Chan, L. Sun, X. Su, J. Zhang and A.A. Berlin, Appl. Spectros. 58, 1401-1407, 2004