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Research Article

Evaluation of SERS labeling of CD20 on CLL cells using optical microscopy and fluorescence flow cytometry

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Abstract

Immunophenotyping of lymphoproliferative disorders depends on the effective measurement of cell surface markers. The inherent light-scattering properties of plasmonic nanoparticles (NPs) combined with recent developments in NP design may confer significant advantages over traditional fluorescence probes. We report and evaluate the use of surface-enhanced Raman scattering (SERS) gold NPs (AuNPs) conjugated to therapeutic rituximab antibodies for selective targeting of CD20 molecules. SERS AuNPs were prepared by adsorbing a Raman-active dye onto the surface of 60 nm spherical AuNPs, coating the particles with 5 kDa polyethylene glycol, and conjugating rituximab to functional groups on polyethylene glycol. The effective targeting of CD20 on chronic lymphocytic leukemia cells by rituximab-conjugated SERS AuNPs was evaluated by dark-field imaging, Raman spectroscopy, and flow cytometry with both competitive binding and fluorescence detection procedures. Evidence of CD20 clustering within approximately 100 nm was observed.

From the Clinical Editor: This study discusses the use of surface enhancement Raman scattering (SERS)-based plasmonic gold nanoparticles, which can be used for cell specific labeling. In this example rituximab, a commercially available CD20 humanized monoclonal antibody is used. Dark field imaging, Raman spectroscopy and flow cytometry was utilized to demonstrate the sensitive labeling capability of these gold nanoparticle based hybrid nanodevices.

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Key words: Surface-enhanced Raman spectroscopy; Gold nanoparticles; Rituximab; Chronic lymphocytic leukemia; CD20

Surface-enhanced Raman scattering (SERS) nanoprobe have been developed for biological detection.¹ To advance this technology to the clinic, it will be necessary to characterize the chemical design of the nanoprobe and to test their ability to recognize clinical samples. Currently the evaluation of cell surface protein expression using fluorescence-based sensing is the predominant strategy for the diagnostic investigation of lymphoproliferative disorders.² The use of fluorescent probes introduces several constraints for which surface-enhanced Raman scattering gold nanoparticles (SERS AuNPs) have been proposed by many as an alternative immunophenotyping solution. SERS probes are not susceptible to photobleaching upon repeated or prolonged illumination^{3,4}; histological stains

that can quench or interfere with fluorescence signals do not interfere with SERS probes, and the narrow bandwidths of SERS vibrational spectra increase the potential for multiplexed marker detection over what can be envisioned with fluorescence.⁵ AuNPs have light-scattering characteristics that enable their use both as SERS substrates to enhance the Raman scattering intensity from reporter molecules incorporated in the structure of a SERS probe,^{6–8} and also as cell labels for dark-field microscopy because of their intense Rayleigh scattering.^{9–11}

SERS immunophenotyping initially focused on targeting the expression of a single cell surface protein,¹² and the field has significantly expanded. Silica,^{13,14} polymer,¹⁵ and protein¹⁶ coatings have been utilized to overcome a number of potential disadvantages of the SERS nanoprobe approach, including irregular SERS intensity of the probes, limited solubility in aqueous solution, and biocompatibility. Different targeting moieties have been employed to direct the SERS probes to their intended targets including antibodies,^{17,18} affibodies,¹⁹ peptides,²⁰ and folic acid.²¹ The full potential of SERS immunophenotyping lies in multiplexed detection, which has been demonstrated for a

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number of surface receptor combinations,^{22,23} and in vivo targeting^{6,24} has been investigated.

This article illustrates a set of methods to characterize SERS probes for immunophenotyping and to evaluate chemical design and effectiveness in recognizing clinical samples. Our example system is chronic lymphocytic leukemia (CLL), which is a typical model of circulating cancer and a good target for single cell analysis. We focus on the targeting of SERS probes to CD20, a transmembrane phosphoprotein expressed in conjunction with a range of B-cell lymphoproliferative disorders.²⁵ The particles are targeted using rituximab, a US Food and Drug Administration–approved chimeric antibody composed of murine anti-CD20 binding regions fused to human IgG,²⁶ typically used in the treatment of CLL and non-Hodgkin's lymphoma. This study is conducted using CLL patient derived leukocyte samples that are prepared in the same manner as for fluorescent flow cytometry immunophenotyping so as to generate the most clinically relevant conditions for evaluating SERS AuNP targeting. The advantages and disadvantages of this reported SERS nanoprobe application to patient derived cells are described.

Each component in the preparation of the SERS probes herein was chosen to produce markers with efficient performance for our application. AuNPs with 60 nm diameter were chosen as the SERS substrate, because they have been demonstrated as providing a large SERS enhancement for use with a 632.8 nm laser.²⁷ An additional advantage of using 60 nm diameter Au is that this size is not associated with significant levels of nonspecific uptake by cells.²⁸ The 5 kDa poly(ethylene glycol) (PEG) coating used to prepare the SERS probes is used widely by others for NP stability,²⁹ water solubility,³⁰ and prevention of nonspecific association with proteins and cell surfaces,^{31,32} and is well characterized.^{6,10,30,33} The choice of 5 kDa specifically however, is an area where our NP design could be further optimized. Rituximab and other therapeutic antibodies have been successfully applied elsewhere for non-SERS-based targeting of AuNPs.^{34,35} The Raman signal intensity, polymer coating thickness, and conjugation to monoclonal antibodies are characterized to understand the structure, and confirm the quality of the SERS AuNPs produced for study.

Rituximab-SERS AuNPs were found to be compatible with flow cytometry, and the specificity of their CD20 binding was evaluated using this technique. The aggregation state of the SERS particles under in vitro conditions is also observed using dark-field microscopy, because plasmon shifts are recorded as changes in the color of the particles and could serve as a medium through which to study clustering of receptors on the cell surface. Flow cytometry also serves as a benchmark against which to compare bulk cell suspension Raman spectroscopy of SERS probe targeted cells.

Methods

The following materials were used with no further purification: 60 nm diameter AuNPs and anti-human Fc antibodies bound to 5 nm AuNPs were purchased from Ted Pella (Redding, California). Malachite green isothiocyanate (MGITC) was supplied by Invitrogen (Burlington, Ontario, Canada). Both 5 kDa α -methoxy- ω -mercapto poly(ethylene glycol) (CH₃O-

PEG-SH) and α -carboxy- ω -mercapto poly(ethylene glycol) (COOH-PEG-SH) were obtained from Rapp Polymere GmbH (Tübingen, Germany). 1-ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), and microformat bicinchoninic acid (BCA) assay came from Thermo Fisher Scientific (Rockford, Illinois). Goat anti-human Fc fluorescein isothiocyanate (FITC) antibodies were from Sigma-Aldrich (Oakville, Ontario). FITC-anti-CD20 came from Beckman Coulter (Mississauga, Ontario). The use of blood samples for research was approved by the Research Ethics Board at the University of Toronto.

Preparation of rituximab-conjugated PEGylated surface-enhanced Raman scattering (SERS) AuNPs

Aqueous solution of MGITC dye was added to stirring Au colloid to a concentration of 33 nM. 5 kDa heterobifunctional CH₃O-PEG-SH was added dropwise from aqueous solution to SERS-active AuNPs, and the mixture was allowed to react at 20°–25°C for 30 minutes. Aqueous COOH-PEG-SH was added to the stirring particles to include reactive groups for conjugation to monoclonal antibodies. Aqueous EDC and sulfo-NHS were added to the stirring SERS NP mixture, as described elsewhere^{36,37} and as discussed in detail in the Supplementary Materials (available online at <http://www.nanomedjournal.com>). Excess cross-linking agents were removed and rituximab antibodies (Roche, Mannheim, Germany) (1.78 mM) added to activated SERS NP suspension and allowed to react for 3.5 hours at room temperature. To provide negative controls, the same procedure was adopted to prepare unconjugated SERS NPs and SERS probes bound to nonspecific murine IgG1 antibodies (Figure 1, A).

Preparation of CLL cells and cell labeling with SERS AuNP-rituximab conjugates

Peripheral blood samples were obtained from CLL patients during clinical follow-up as described in Supplementary Materials. The cells were suspended in phosphate-buffered saline (PBS) plus 1% (v/v) fetal calf serum. SERS AuNP labeling was performed by incubation of cells with NP-rituximab conjugates at high (5.6 pM) or low (1.4 pM) concentration where noted in PBS at room temperature for 30 minutes.

NP characterization

Ultraviolet–visible (UV–vis) absorption spectroscopy was carried out on a Cary 5000 UV–vis spectrometer (Varian, Palo Alto, California) from aqueous suspensions of NPs placed in 1 cm pathlength cuvettes.

The hydrodynamic size of SERS AuNPs was assessed after each functionalization step via dynamic light scattering (DLS). DLS measurements were collected on a DynaPro/Protein Solutions DLS machine (Wyatt Technologies, Santa Barbara, California), and data processed using Dynamics software version 6.7.1.

To further confirm successful PEG coating of the SERS AuNPs, scanning electron microscopy (SEM) was used (Hitachi S-5200; Hitachi, Tokyo, Japan). To prepare samples of SERS AuNPs for SEM, 15- μ L drops of NP suspensions were placed on carbon-coated copper grids and air dried. Images were taken at 30 kV using 500,000 $\times$ magnification.

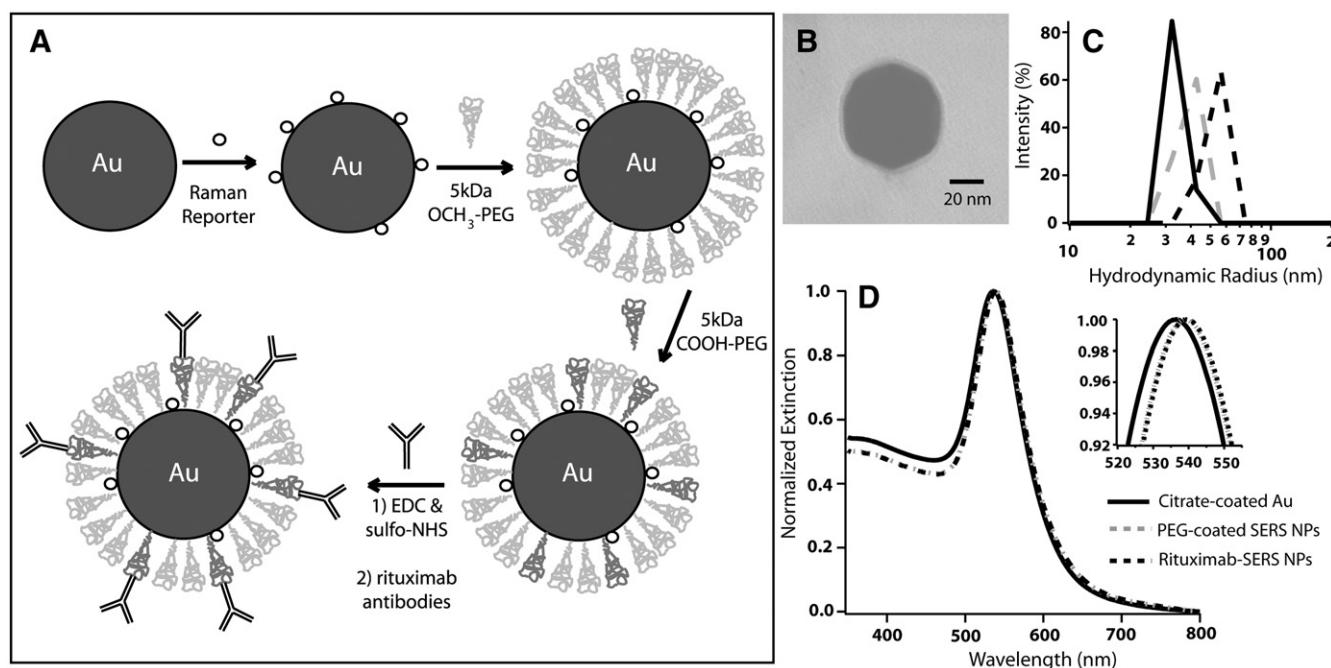


Figure 1. (A) Schematic illustration of the preparation of rituximab-conjugated SERS gold nanoparticles (AuNPs). (B) SEM image representative of rituximab-SERS AuNPs. (C) Dynamic light scattering histograms indicate an increase in particle hydrodynamic radius following each surface functionalization step. (D) Visible absorption spectra of SERS AuNPs. Inset: a 2-nm shift in the particle plasmon absorption occurred upon PEGylation, and the absorbance maximum did not shift further following conjugation to rituximab antibodies.

Quantification of rituximab antibodies conjugated to SERS AuNPs

Three different methods were used to estimate the average number of rituximab antibodies conjugated to each SERS AuNP. Detailed procedures are provided in Supplementary Materials. Goat anti-human Fc FITC antibodies were used to label rituximab-conjugated and control SERS NPs for comparison, and fluorescence measurements were collected using a spectrofluorophotometer (Shimadzu RF-5301PC; Columbia, Maryland) with RF-530XPC software, in 1 cm pathlength cuvettes.

For labeling in transmission electron microscopy (TEM) images, rituximab-conjugated and control SERS AuNPs were each incubated with secondary anti-human Fc antibodies bound to 5-nm AuNPs for 2 hours at room temperature. TEM images were collected using a Hitachi H-7000 TEM at 75 kV with 50,000 $\times$ magnification.

BCA assay was used to measure protein concentration in rituximab-conjugated AuNP suspension as per assay instructions.

Dark-field microscopy

Dark-field light scattering images were collected using a Nikon TE2000 microscope equipped with oil immersion condenser and objective (100 $\times$, 0.5–1.25 NA) (Nikon Instruments, Melville, New York). Details of image analysis procedures are provided in Supplementary Materials.

SERS microspectroscopy

The same inverted microscope was used to focus a 632.8 nm HeNe laser (10 mW; CVI Melles Griot, Albuquerque, New

Mexico) onto drops of cell suspension through a 40 $\times$ (0.6 NA) objective. A notch filter ($\lambda = 632.7$ nm, full width at half-maximum = 23.8 nm) was used to prevent Rayleigh scattering from entering the monochromator. Raman scattered light was collected by a Peltier cooled charge-coupled device detector with 1340 $\times$ 400 pixel array (PIXIS BR400; Princeton Instruments, Acton, Massachusetts) using WinSpec/32 software. Spectra of cell samples were collected from drops of cell suspension on glass coverslips using 90 second integration time.

Flow cytometry

Cells were incubated with either unconjugated rituximab or rituximab-conjugated SERS AuNPs for 30 minutes at room temperature. Secondary staining was then performed with two fluorescent antibodies: Fc-specific FITC-anti-human IgG was used to detect rituximab conjugates bound to the cells; and FITC-anti-CD20 was used to measure the competitive blocking by the SERS-rituximab binding to CD20. Cells stained with secondary antibodies alone were used as controls. For each sample, at least 5000 events were acquired on a Cytomics FC500 flow cytometer (Beckman Coulter), and data analysis was performed using CSP software (Beckman Coulter) and FlowJo (Treestar, Ashland, Oregon).

Results

Characterization of rituximab-SERS AuNPs

UV-vis spectroscopy confirmed the monodispersity of the SERS AuNPs after each functionalization step (Figure 1, D). After addition of aqueous thiolated PEG to the SERS AuNP

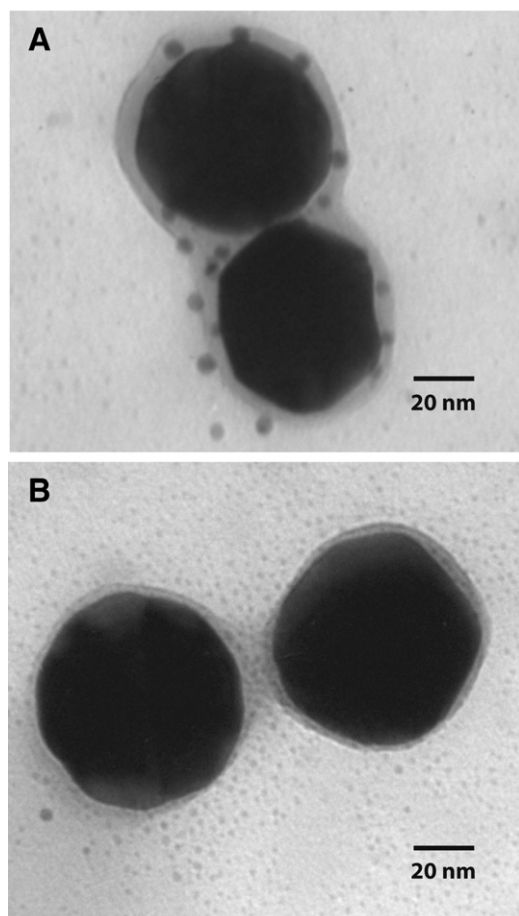


Figure 2. TEM images of SERS AuNPs incubated with anti-human Fc 5 nm Au secondary antibodies for identification of rituximab primary antibodies on NP surfaces. **(A)** Rituximab-conjugated SERS AuNPs. **(B)** SERS AuNPs with PEG coating only.

suspension, the formation of a polymer layer was confirmed through a 2 nm red shift in the localized surface plasmon resonance (LSPR) absorbance of the NP suspension from 534 nm to 536 nm, and the peak location remained consistent following conjugation to rituximab antibodies. An absorption peak did not appear in the 600 to 700 nm region, which would have indicated the formation of particle aggregates. DLS measurements further confirmed SERS AuNP functionalization and monodispersity (Figure 1, C). The stock citrate-coated AuNPs used to prepare SERS probes in this study had a hydrodynamic radius of 33 ± 0.2 nm. Following coating with PEG, this radius increased to 39 ± 0.8 nm, and finally to 55 ± 1.6 nm once conjugated to rituximab antibodies. Particle diameter was confirmed by measuring particles in a series of TEM images (Figure 1, B). Mean diameter using this method was determined to be 29 ± 2 nm.

Quantification of antibody conjugation

FITC fluorescence from NPs suspensions incubated with anti-human Fc secondary antibodies gave an average value of 80 ± 30 antibodies per rituximab–SERS AuNP. In TEM imaging, anti-human 5 nm diameter Au secondaries appear as dark-gray spots within the lighter gray PEG and protein corona

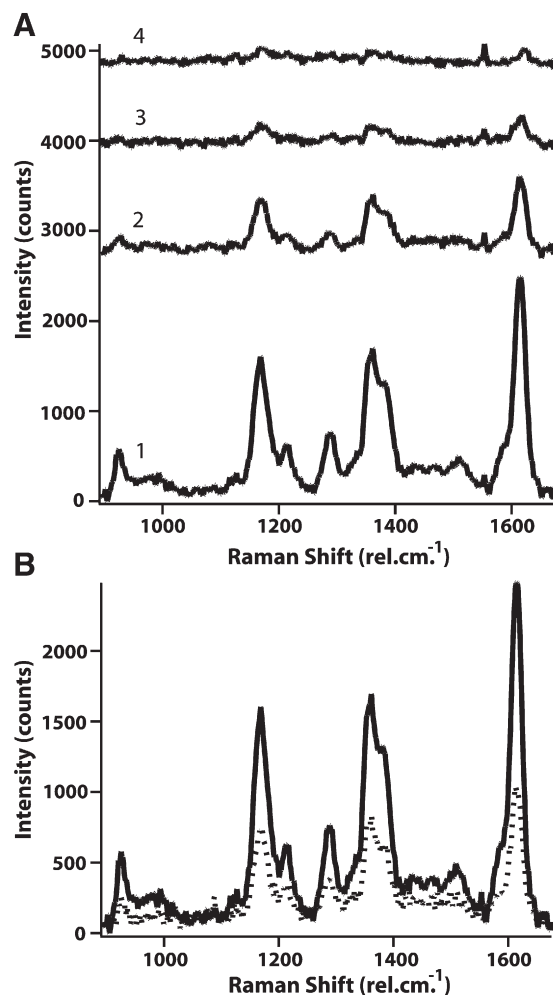


Figure 3. SERS spectra of cell suspensions in PBS following incubation with SERS AuNPs. **(A)** SERS intensity from targeted cell samples stained with high (1) and low (2) concentrations of rituximab–SERS AuNPs (5.6 pM and 1.4 pM, respectively), in contrast with negative controls using murine IgG1 nonspecific SERS AuNPs (3) and SERS AuNPs with PEG coating only (4). **(B)** SERS intensity in competitive binding experiments. Cell samples stained with high concentration of rituximab AuNPs (black); cells prestained with unlabeled rituximab before treatment with high concentration SERS probes (dashed). SERS peak assignments are available in Supplementary Materials.

surrounding the dark 60 nm AuNPs, and bind specifically to SERS NPs conjugated to rituximab (Figure 2). This estimation method gave a value of 30 ± 5 rituximab antibodies per 60 nm SERS AuNP. The micro-BCA assay gave values for total protein concentration in rituximab-conjugated AuNPs suspension, and this antibody count was averaged over the approximate number of SERS AuNPs present, yielding a value of 22 ± 0.6 rituximab antibodies bound to each SERS probe.

Evaluation of CD20 targeting of CLL cells using SERS spectroscopy

The specific MGITC SERS NP spectrum was detected in cell samples labeled with rituximab–SERS AuNPs at high and low concentrations (Figure 3, A). Positively targeted cell samples were clearly distinguishable from negative controls that used

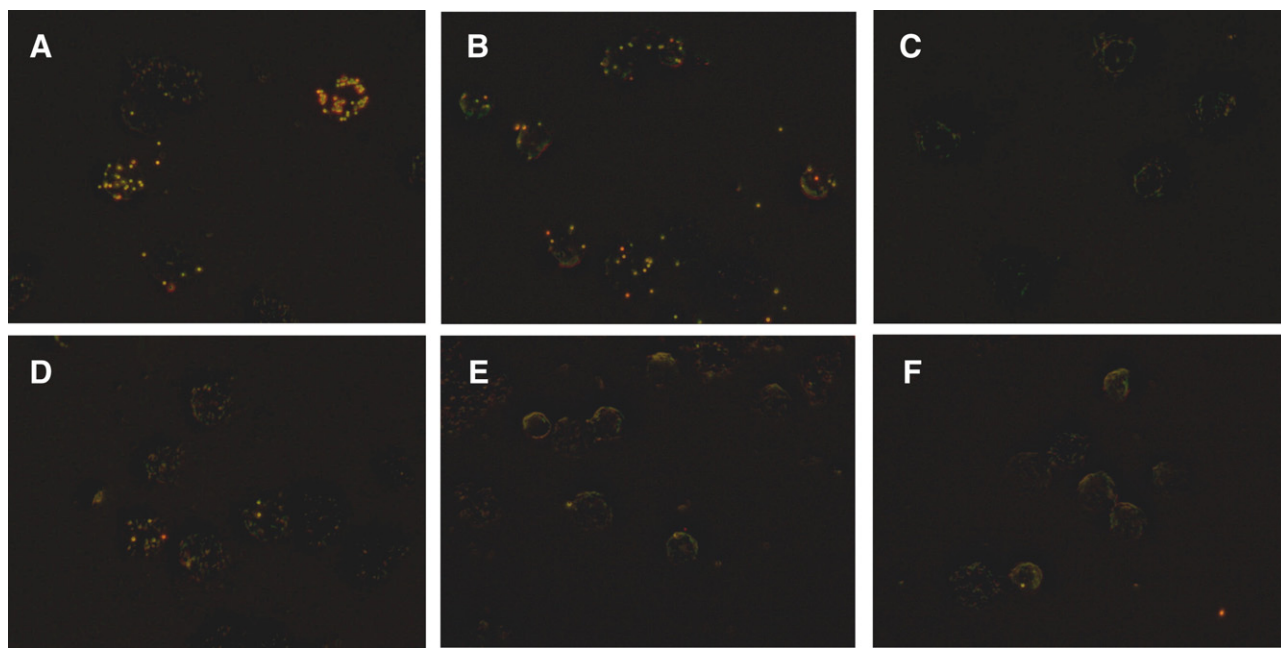


Figure 4. Representative dark-field scattering images of CLL cells labeled with SERS AuNPs (100 $\times$ magnification). The blue channel has been subtracted from original RGB images, and brightness increased to show cell location. Top row: CLL cells subjected to varying SERS AuNP treatments. **(A)** High concentration of rituximab-conjugated SERS AuNPs; **(B)** low concentration of rituximab-SERS AuNPs; **(C)** nonspecific-isotype IgG1-SERS AuNPs. Bottom row, rituximab blocking: images of CLL cells treated with un conjugated rituximab before corresponding SERS AuNP labeling. **(D)** High concentration of rituximab-conjugated SERS AuNPs; **(E)** low concentration of rituximab-SERS AuNPs; **(F)** nonspecific-isotype IgG1-SERS AuNPs. Details of image processing and raw images are provided in Supplementary Materials.

murine IgG1 nonspecific SERS AuNPs or SERS AuNPs with PEG coating only (Figure 3, A). Competitive binding experiments were performed whereby CD20 binding sites were blocked using unlabeled rituximab before labeling with SERS AuNPs. A marked reduction in SERS signals was observed for those CD20 blocked samples in comparison to the nonblocked samples of equivalent AuNP labeling (Figure 3, B). No distinction could be made between the CD20 blocked and nonblocked cells treated with nonspecific IgG1-SERS NPs (not shown).

Evaluation of CLL cell labeling using dark-field microscopy

Dark-field imaging of fixed cell cytospin slides was used to further confirm the cell labeling by rituximab-SERS AuNPs. Cell labeling densities as observed using dark-field microscopy strongly correlated with observations made using SERS spectroscopy (Figure 3). Positively labeled cell samples using different concentrations of rituximab-bound SERS probes (Figure 4, A and B) exhibited corresponding densities in CD20 labeling. Images of cells treated with nonspecifically targeted SERS NPs did not display significant SERS AuNP labeling (Figure 4, C) and can be clearly differentiated from positively targeted samples. Competitive binding experiments involved the blocking of CD20 binding sites with unlabeled rituximab before incubation with SERS AuNPs. Rituximab-SERS AuNP binding was markedly reduced by the CD20 blocking (Figure 4, D and E). Little or no cell labeling was observed by nonspecific IgG1-SERS AuNPs, whether or not the CD20 epitopes were masked with unlabeled rituximab (Figure 4, C and F). The differences in

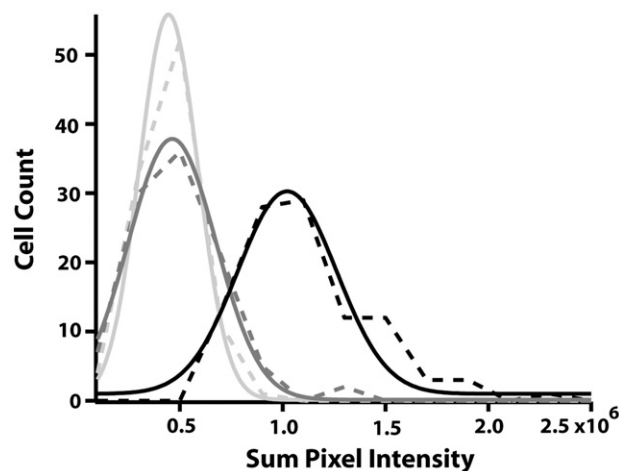
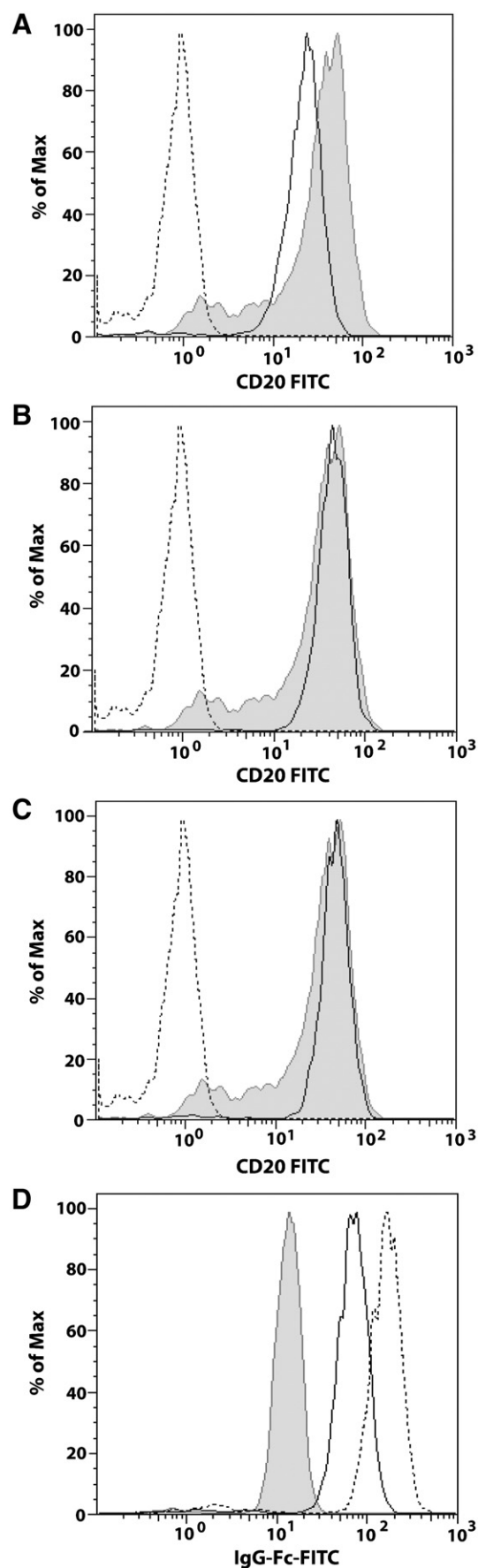


Figure 5. Distribution of the sum of pixel intensities corresponding to cell location in dark-field images. Dashed lines show raw data; Gaussian fits are represented by solid lines. Cells treated with a high concentration of rituximab-SERS AuNPs (black); competitive binding treatment whereby cells were blocked with unlabeled rituximab antibodies before treatment with a high concentration of rituximab-SERS AuNPs (dark gray); cells treated with nonspecific IgG1-SERS probes (light gray).

cell labeling densities between NP treatments were quantified by plotting the distribution of the sum of pixel intensities corresponding with the location of individual cells in a series of dark-field images (Figure 5). For each of three different AuNP



treatments, respectively, a series of 100 cells were imaged using identical microscopy and imaging settings to complete the analysis. The pattern observed was consistent with visual observations, in that cells treated with rituximab-bound SERS probes were significantly more intense than those treated with nonspecific IgG particles, or subjected to competitive binding experiment (Figure 5).

NP counting and cell labeling intensities in dark-field imaging

The NPs are expected to Rayleigh scatter light preferentially at the location of their LSPR, which corresponds to 536 nm for 60 nm diameter functionalized AuNPs, and hence should appear as green-gold in the dark-field images, while the LSPR of clustered or aggregated species should red shift. Image analysis software (Photoshop CS3; Adobe Systems, San Jose, California) was used to objectively isolate and count these separate populations. Values calculated by the software counting procedure were compared to manual particle counts, and the percentage difference between them was calculated. For a series of eight images of high volume anti-CD20 SERS AuNP cell staining at 100 $\times$ magnification, there was approximately a 3:1 ratio of green to red particles as recorded by automated counting, while this ratio was identified as 10:1 by manual counting. The average percentage difference between sets of values obtained from automated and manual counting methods is 33% for green particles and 78% for red particles. The variation arises from the ability of the automated procedure to objectively differentiate colors, while the manual count is more efficient at discerning individual particles located in clusters in the cell samples.

Flow cytometric analysis

The specificity of B-cell labeling by rituximab-SERS probes was further evaluated using flow cytometric assays. Fluorescence detection was undertaken using anti-human IgG (Fc-specific) antibody and antibody to CD20. A reduction of FITC-CD20 fluorescence (Figure 6, A) was observed from cell samples first blocked with unlabeled rituximab before treatment with rituximab-SERS AuNPs, although the decrease in fluorescence was not as significant as for samples blocked with unlabeled rituximab (Figure 6, A). By contrast, no decrease in anti-CD20 fluorescence was observed following prestaining with either unconjugated SERS AuNPs (Figure 6, B) or nonspecific isotype IgG1-SERS AuNPs (Figure 6, C).

Secondary antibody fluorescence detection of rituximab-conjugated SERS AuNPs was conducted using anti-human Fc FITC antibody, specific for the Fc fragment of rituximab. Significant binding of the secondary was observed on cells with

Figure 6. Flow-cytometric assessment of SERS AuNP-rituximab binding. (A) Changes in fluorescence intensity of anti-CD20 FITC antibody in cells prestained with unconjugated rituximab (dashed) or rituximab-SERS AuNPs (solid), in comparison to cells stained directly with antibody to CD20 (gray). No blocking was observed following the prestaining with either (B) unconjugated SERS AuNPs or (C) IgG1-SERS AuNPs. (D) Detection of rituximab-SERS AuNPs by secondary staining with an anti-human IgG (Fc-specific) FITC antibody. Cells prestained with unlabeled rituximab (dashed); rituximab-SERS AuNPs (solid); control cells without primary staining (gray).

the primary binding of either unconjugated rituximab or rituximab-conjugated SERS AuNPs (Figure 6, D). The fluorescence from both samples was markedly enhanced from the baseline obtained using anti-human FITC antibody alone (Figure 6, D).

Discussion

In this study we demonstrated rituximab mediated CD20 targeting of SERS AuNPs, and the confirmation of this targeting using Raman spectroscopy, dark-field microscopy, and flow cytometry analysis. It is significant to note that cell surface protein labeling using SERS probes was not only compatible with flow cytometry immunophenotyping but could also have its efficacy evaluated using this technique.

The Raman reporter used in this study, MGITC, was electrostatically adsorbed to the surface of colloidal AuNPs, where LSPR enhanced the intensity of the dye spectrum by approximately four orders of magnitude. The magnitude of the enhancement via electromagnetic enhancement mechanism³⁸ is consistent with previously reported values for monomer particles,^{39,40} and details of the enhancement factor calculation are provided in Supplementary Materials. A 2 nm shift in the plasmon peak of the particles (Figure 1, D) and an approximately 10 nm increase in their hydrodynamic radius (Figure 1, C) were sufficient to confirm the formation of a PEG layer surrounding the SERS AuNPs, although alternative approaches have been used elsewhere.^{11,24}

Bioconjugation of the PEGylated SERS AuNPs occurred through the formation of amide bonds between carboxylic acid functionalities of the PEG coating, and available ϵ -amine groups of lysine residues, or N-terminal amines of the rituximab antibodies.³⁷ A disadvantage of this method is that the orientation of antibody binding is uncertain, so that some of the antibodies to CD20 were probably oriented in a configuration that reduced the affinity for their CD20 targets. This fact may not limit the efficacy of anti-CD20 SERS AuNPs as surface receptor labels, however, as multiple antibodies on average bind each NP, increasing the likelihood that at least one targeting moiety will be bound in the correct orientation for cell surface labeling. Multivalent targeting agents, including those for targeting rituximab, have also generated interest because of increased potency over monovalent therapeutic antibody constructs.⁴¹

Quantification of the number of antibodies bound to the surface of each SERS AuNP was not trivial, and therefore several methods were employed in conjunction. Although the absorbance and emission maxima for FITC antibodies used in secondary fluorescence labeling (located at 494 nm and 521 nm, respectively) overlap with the AuNP plasmon, the 10 nm separation of the fluorophores from the NP surface prevents significant fluorescence quenching.⁴² Secondary 5 nm immunogold antibody labeling of rituximab-conjugated SERS NPs^{10,35} provided a lower estimate than fluorescence labeling for the average number of antibodies bound per SERS probe. Steric hindrance is introduced by the 5 nm AuNPs that could prevent anti-human Fc secondary antibodies from approaching adjacent rituximab antibodies bound to the 60 nm Au surface. This would not have been a factor in the secondary fluorescence

labeling. Additionally, some 5 nm secondary AuNPs appear to fall outside of the PEG and protein corona in the TEM labeling images (Figure 2, A), which could be suggestive of a nonspecific association of 5-nm particles with 60 nm Au during the drying process of the sample deposited on to the TEM grid. This is probably not the case, however, as not a single 5 nm Au NP was associated with the 60 nm SERS Au in a series of 55 particles in control images whereby SERS Au with PEG coating only were treated with 5 nm Au secondary IgG particles.

A different approach was taken by measuring total protein concentration using a colorimetric micro-BCA assay. The BCA assay was chosen as it is highly sensitive, it yields linear calibration curves, and its color development is not as susceptible as that of other protein assays to differences in sequence between proteins. Absorbance is measured at 562 nm and increases in intensity with increasing protein concentration. Because the maximum absorbance wavelength of the assay at 562 nm overlaps with the intense 60 nm AuNP plasmon peak, the particles were removed from the assay solution before taking measurements. It has been reported elsewhere that the presence of the NPs causes nonspecific color development.^{11,43} The BCA assay is sensitive to the presence of thiols in solution, and it is possible that the thiol-terminated PEG coating the NPs could be falsely increasing the protein concentration values. To control for this issue, the assay was also conducted on a suspension of PEG-coated particles that were not bound to antibodies.

To assess SERS probes bound to CD20 on CLL cells, SERS spectroscopy was used to detect the MGITC fingerprint spectrum from drops of cell suspension. The MGITC SERS spectrum was clearly observed from positively targeted cell samples; however, a linear relationship between this spectral intensity and the amount of anti-CD20 SERS NPs was not immediately evident (Figure 3, A). However, if the SERS intensity obtained from the sample treated with nonspecific IgG1 isotype-conjugated SERS AuNPs was used as the background intensity level, subtracting this value from each of the positive labeling treatments yields a 1:4 intensity ratio between the treatment groups. This intensity ratio correlates linearly with the 1:4 NP volume ratio used in the low and high anti-CD20 SERS AuNP concentrations, respectively. Flow cytometry results were indicative of a lack of CD20 receptor saturation by the concentrations of SERS AuNPs used in these staining procedures. As such, it would be expected that SERS intensity should rise roughly linearly with an increase in the number of anti-CD20 NPs used in each cell sample, consistent with the trend observed in the SERS spectra.

Competitive binding experiments were performed to confirm the specificity of rituximab–SERS AuNPs binding to CD20 targets. When CD20 receptors were blocked using unconjugated rituximab antibodies, a marked reduction in intensity compared to the equivalent unmasked samples was recorded (Figure 3, B), thus indicating the specificity of NP binding. SERS intensity collected from CD20 blocked and unblocked cells incubated with murine IgG1–SERS AuNP controls were equivalent, which further indicates that SERS intensity obtained from the isotype control samples may be treated as low-level background intensities (not shown). This background signal was a result of either nonspecific association of the particles with cells in the

sample, or a remainder of residual SERS particles in suspension after extensive washing of the cell samples.

Evaluation of absolute receptor expression using flow cytometry requires careful calibration of the intensity of the different fluorophores used and the response from the flow cytometer to these, in addition to objective data analysis; protocols have been developed for this purpose.^{44,45} Likewise, relative and absolute receptor expression could be obtained using SERS techniques, but the way in which this is evaluated will differ from fluorescence methods. A disadvantage of the use of SERS probes for immunophenotyping is the possibility of nonlinear signal increases with increasing particle binding density as a result of surface plasmon coupling between neighboring particles. This would result in nonlinear increases in SERS intensity with receptor number. The detailed design of quantitative SERS immunophenotyping procedures is an important subject for further study.

Light scattering dark-field images of cytospin slides were taken to ascertain the spatial location of the SERS AuNPs within labeled cell samples and to further evaluate the relative degrees of cell labeling by functionalized probes (Figure 4). The dense cells scatter light strongly and appear blue-white in the images, whereas SERS NPs intensely Rayleigh-scatter at the wavelength corresponding to their LSPR at 536 nm, resulting in monomer particles appearing green-gold in dark-field images.⁴⁶

Some red light scattering species are visible in the dark-field images and are probably aggregates or clusters of particles, which could have been introduced either during the NP functionalization process, or during subjection of the particles to *in vitro* biological conditions causing a shift in their LSPR.^{47,48} The SERS particle UV–vis spectrum does not show an absorbance at wavelengths >570 nm, which would be characteristic of the formation of particle aggregates. Taken together, these observations indicate that the plasmon shift observed in dark-field images is due to particles binding proximal CD20s on the cell surface resulting in observation of receptor clustering within the separation necessary (<50 nm) to cause the plasmon shift.⁴⁹

A potential advantage of immunophenotyping with plasmonic particles as opposed to fluorophores is the potential to study receptor distributions within regions of tens to hundreds of nanometers separation on cell surfaces. Although fluorescence technologies enable visualization of receptor location, and areas that fluoresce intensely are indicative of a high density of the surface protein being labeled, information about the spacing and density of the receptors is constrained by the diffraction limit of visible light. Super-resolution fluorescence microscopy techniques are being increasingly adopted to address such questions⁵⁰; however, such imaging systems are currently not widely available. Plasmonic metal NPs exhibit distance dependent plasmon coupling in a predictable manner, thus red shifting their LSPR wavelength and providing information about the distance between adjacent particles, which can be collected using the simple, inexpensive dark-field microscopy technique. A disadvantage of plasmonic NPs for such distance studies is sensitivity to particle aggregation before going down on the cell surface, which shifts the LSPR wavelength independent of cell binding. As discussed above, significant aggregation of the SERS probes in this study was not evident before their use in cell studies, and therefore color changes observed are probably the result of binding density. To

ensure validity of surface receptor density studies using plasmonic particles, it is important to have controls in place to ensure that aggregation does not occur before surface binding.

Flow cytometry further confirmed the effective targeting of rituximab-conjugated SERS AuNPs to CD20 by assessing the retention of functionalized AuNPs by CLL cells. An increase in secondary fluorescence intensity was observed from cells first treated with rituximab-conjugated probes (Figure 6, D). Although the enhancement was not as significant as for unlabeled rituximab, it was markedly increased relative to the baseline level obtained using secondary antibody alone.

SERS probe binding specificity was investigated using a flow-cytometric competitive assay in which CLL cells were stained with a fluorescent antibody to CD20 following incubation with rituximab-conjugated SERS AuNPs, resulting in a subsequent decrease in anti-CD20 fluorescence (Figure 6, A). This effect can be attributed to the blocking of CD20 epitopes on the cell surface by the rituximab. Similar to the findings from SERS spectroscopy, the rituximab–SERS AuNPs did not saturate CD20 binding sites to the same extent as pure rituximab, as indicated by the complete blocking of CD20 fluorescence following the incubation of cells with unconjugated antibody. In contrast, there was no decrease in anti-CD20 fluorescence following preliminary staining with controls of either nonspecific isotype IgG1 control (Figure 6, C) AuNPs or unconjugated AuNPs (Figure 6, B).

The competitive binding assays provide strong evidence for the effectiveness and specific targeting of rituximab-conjugated SERS AuNPs to CD20. The disparity in targeting efficiency compared with free rituximab could be attributed to a failure to achieve CD20 saturation with the concentrations of rituximab conjugates used in this experiment. Alternatively, this effect may be an indication of steric hindrance at the cell surface caused by the blocking of neighboring CD20 binding sites by bulky 60-nm AuNPs. Receptor saturation dynamics using large SERS NPs should be studied further, to overcome potential disadvantages of SERS nanoprobe compared with the use of molecular reporters.

A comparison of SERS technologies to conventional flow cytometric immunophenotyping was provided for leukemia detection and diagnosis, and may be useful in evaluating the SERS nanoprobe's application to other systems. An important subject for future study is an understanding of the relative and absolute expression levels of cell surface proteins that takes into account the effects of plasmon shifts from particles binding separately but near each other, the effects of particle-particle contacts at the surface, and any resulting SERS enhancement above that of monodispersed SERS particles. It would also be valuable to construct SERS nanoprobe that not only report the presence of antigen because of immunochemistry but also provide enhanced spectroscopic characterization of the biological material within the plasmonic field of the bound NPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2012.04.003>.

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