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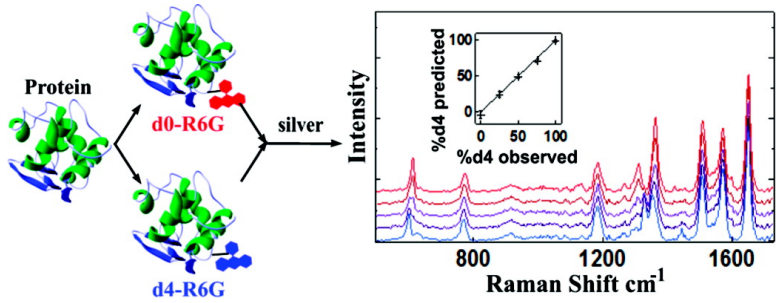

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Detection and Relative Quantification of Proteins by Surface Enhanced Raman Using Isotopic Labels

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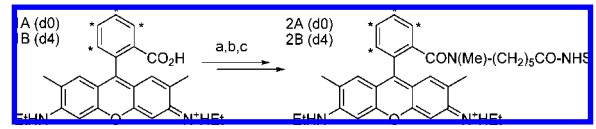
Accurate and precise protein quantification remains an important fundamental methodology and represents a critical component in identifying biomarkers and early detection of diseases.[1] The capacity to quantify relative changes in proteins from complex biological samples is a central challenge to understanding the response of protein expression to genomic or environmental factors. Surface Enhanced Resonance Raman Spectroscopy (SERRS) has proven to be a useful spectroscopic tool for diagnostic applications,[2–5] explosive detection,[6] monitoring events in enzyme catalysis,[7] detection of metabolites,[8] and detection of bioanalytes during capillary electrophoresis.[9] However, SERRS suffers from variability in enhancement of Raman intensity depending upon the nanomorphology of the substrate (typically silver colloids), thereby affecting the reproducibility of the measurement. In our previous work, we have demonstrated the improved reproducibility and accuracy of quantitative SERRS measurements obtained using isotopomeric rhodamine 6G (R6G) probes over a useful analyte concentration range (picomolar to micromolar) of analytes.[10] Herein, we demonstrate for the first time that these dyes may be transformed into biomolecular labeling reagents and used for SERRS based quantification of labeled proteins in a polyacrylamide matrix.

The sensitivity of SERRS can rival that of fluorescence, a standard for optical detection and quantification of antibodies and nucleic acids.[11] Advantages of SERRS include the narrow band widths and chemical fingerprint information of Raman spectral lines, as well as the improved quantification capability of the isotopic SERRS approach.[10] The SERRS of suitable dye probes such as R6G adsorbed on roughened metallic nanostructures dominates over other Raman scattering processes and enables the detection of dilute analytes in mixtures.[12] These chemical features provide suitable enhancements of the Raman signatures for the dye to allow detection in the background of matrix immobilized protein. By using isotope-encoded dyes for internal standardization of SERRS one should be able to quantify protein concentration with unprecedented accuracy.

In order to establish the above strategy by incorporating isotope-encoded Raman labels into proteins, a method for synthesis of modified R6G was pursued through modification of the carboxylic functional group (Scheme 1). Previous efforts showed that the tetradeuterio-R6G based upon 1A provided reliable differences in SERRS signature and enabled quantification.[10,13] Using this pattern of hydrogen substitution, the precursor d4-R6G-acid (1B) was obtained from condensation of 3-ethylamino 4-methylphenol with d4-phthalic acid. R6G was hydrolyzed under basic conditions to obtain the corresponding acid (1A). Installation of a side chain linker was developed to enable efficient protein conjugation. The xanthene chromophore has a propensity for cyclization under basic conditions.[14] To ensure retention of the xanthene chromophore, a tertiary substituted amide linkage was selected to prevent cyclization at the C7 position under basic conditions required for bioconjugation reactions. A linker was derived by basic hydrolysis of *N*-methyl caprolactam and subsequent esterification of the terminal carboxyl moiety. Amidation of 1A (and 1B) with the linker was followed by ester interconversion by hydrolysis to the free acids and activation to the corresponding NHS esters (2A and 2B). Since lysine tagging is typically done at pH 8.5, we established that the dye linker was stable in aqueous buffer at pH 9.0 for 2 d at room temperature.

Scheme 1[a]



[a] Reagents and conditions: (a) HATU/DIPEA/NHMe$(CH_2)_5CO_2Me$/DMF, 65%; (b) $Ba(OH)_2$/MeOH−H_2O, 45%; (c) (iii) HATU/DIPEA/NHS/DMF, 80%. (NHS = *N*-hyrdoxysuccinimide ester). The sites of deuterium are noted with an asterisk.

To test the feasibility of the bioconjugation approach, protein samples of lysozyme, glycogen phosphorylase, cytochrome c, casein, and ferritin were labeled with the isotopomeric R6G probes and subsequently quantified by SERRS detection: The labeling efficiency for reagents 2A and 2B was first tested using UV−vis spectra of the final dye-labeled samples to standardize the protein and R6G content by the relative absorbance's at 280 and 540 nm, respectively (Supporting Information). Protein labeling on the 200 nmol dye per mg of protein scale gave a labeling efficiency of approximately 5 dye molecules per protein chain but ranged from 0.5 to 10 for different proteins tested and depended upon lysine content of the protein. Lysozyme labeling was further characterized by ESI LC/MS and found to contain one (23% in the d0 sample or 29% in the d4 sample) or two labels (13% d0 or 12% d4).

Examples of the SERRS spectra of the d0/d4 labeled proteins are shown for lysozyme and cytochrome *c* in Figure 1. In all cases, the R6G-labeled protein samples showed identical SERRS spectra to those of the parent R6G labeling reagents. The critical distinctions in the SERRS spectra of the isotopomeric R6G-labeled proteins were also apparent. The Raman fine structure is dominated by the features of the R6G. These colloidal suspensions displayed a limit of detection at an approximately 300 pM protein concentration, which was similar to the level of detection of R6G dye alone. Despite the complexity of surface interactions, the reproducible and predicted SERRS signatures in colloid suspensions with isotopomeric mixtures of the R6G-labeled protein samples established a statistical representation of the dye populations. No features of the

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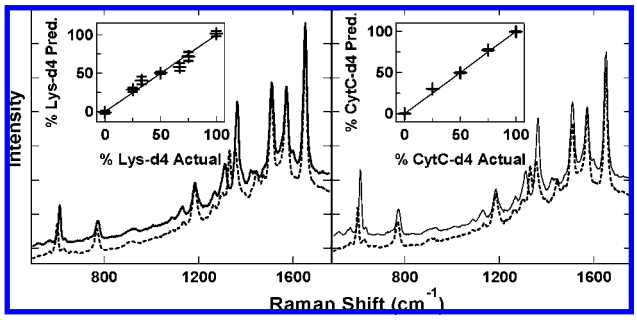

Figure 1. SERRS spectra of (left) 3 nM lysozyme-R6G-d0 (solid) and lysozyme-R6G-d4 (dashed) and (right) cytochrome C-R6G-d0 (solid) and cytochrome-C-R6G-d4 (dashed). The inset calibration curves show the relative amount of d4 labeled protein determined from the ratio of peak areas of the d0 (611 cm^{-1}) peak and the d4 (600 cm^{-1}) peak compared to the known mixture ratio with 95% C.I. error bars. The solid line in the insets represents an ideal slope = 1.

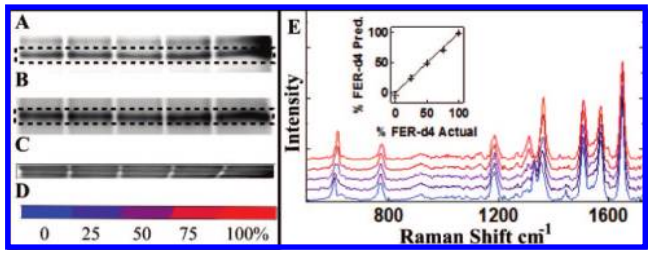

Figure 2. SDS-PAGE gel analysis of 0, 25, 50, 75, and 100% d4-R6G mixtures of isotopomeric R6G-labeled ferritin, loaded with a total of 20 pmol of dye or 0.7 μg protein per lane (see Supporting Information for details of detection). Shown here is the 20 kDa protein band imaged by silver stain (A), fluorescence (B), and SERRS intensity normalized to the most intense signal in the image of the boxed region in A and B (C). (D) A color coded representation of the percent of R6G-d4 label (blue corresponds to 100% R6G-d4 label; red corresponds to 100% R6G-d0-label). (E) Fluorescence baseline subtracted SERRS-spectra of the five mixtures. The inset of E shows the percent d4 determined by least-squares fitting of spectra from the ratio lanes. The error bars represent the 95% C.I. for five measurements taken from each ratio lane.

protein were observed in the Raman spectra, further supporting the dominant role of the dye−nanoparticle exchange interactions as a mechanism for the enhancement.[13]

Polyacrylamide gel based protein separation and quantification play a central role in protein analysis, proteomics, and diagnostics. Spectral detection methods for proteins in gels have been limited to fluorescence. No prior studies have focused on using SERRS detection in gels or applications of isotopic variants for quantitative measurements in such a matrix. To obtain SERRS in a gel matrix, *in situ* formation of silver nanoparticles using a protein silver staining protocol was pursued.[15,16] Ferritin samples labeled with d0- or d4-R6G were mixed in varied ratios and separated on SDS PAGE (Figure 2). The protein bands were imaged first by fluorescence, followed by silver staining and SERRS spectral imaging again using a 514.5 nm laser. The spectral shifts at 600 cm^{-1} were used to calculate the percent isotopic compositions for d0 and d4 R6G corresponding to the relative amounts of the two ferritin samples. The SERRS spectral signature reflects that of the isotopomeric label as shown (Figure 2E). The expected statistical distribution of the two labeled proteins in the PAGE gel format was observed.

Standardization of SERRS measurements by use of isotopomers of the same dye class overcomes many limitations of surface enhanced spectroscopy.[10] Demonstrated here is the feasibility of a bioconjugation approach for quantification of proteins by SERRS detection enabled by isotopic substitution of a Raman active label. Protein Raman signals were not observed in the surface enhanced R6G spectra presumably due to larger SERRS enhancements for the dye−surface interactions. Equally important is the fact that spectra in the immobilized labeled protein accurately reflect the relative molecular content. Formation of an SERRS active silver nanoparticle enhancement agent within a polymeric matrix offers a practical approach to biomolecular detection after capture or separation from biological mixtures. Optimization of the methods to enhance SERRS intensity or minimize fluorescence or other backgrounds offers additional promise.

Future applications of isotopic SERRS-based quantification include comparative proteomics, which would take advantage of labeling protein mixtures from a distinct origin with different isotopomer dye reagents.[17] Additional quantitative biomolecular analyses such as antibody−antigen complexes in semipermeable matrices also represent promising applications for this detection strategy. Highest accuracy measurements are expected when the isotopic ratios are near unity, but preliminary results indicate the method may be applicable with ratios up to a factor of 10.

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Supporting Information Available: Experimental details and spectroscopic data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Unwin, R. D.; Evans, C. A.; Whetton, A. D. *Trends Biochem. Sci.* **2006**, *31*, 473–84.
(2) Stuart, D. A.; Yuen, J. M.; Shah, N.; Lyandres, O.; Yonzon, C. R.; Glucksberg, M. R.; Walsh, J. T.; Van Duyne, R. P. *Anal. Chem.* **2006**, *78*, 7211–7215.
(3) Grubisha, D. S.; Lipert, R. J.; Park, H. Y.; Driskell, J.; Porter, M. D. *Anal. Chem.* **2003**, *75*, 5936–43.
(4) Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884–6.
(5) Wabuyele, M. B.; Vo-Dinh, T. *Anal. Chem.* **2005**, *77*, 7810–5.
(6) McHugh, C. J.; Keir, R.; Graham, D.; Smith, W. E. *Chem. Commun.* **2002**, 580–1.
(7) Moore, B. D.; Stevenson, L.; Watt, A.; Flitsch, S.; Turner, N. J.; Cassidy, C.; Graham, D. *Nat. Biotechnol.* **2004**, *22*, 1133–8.
(8) Ruan, C.; Wang, W.; Gu, B. *Anal. Chem.* **2006**, *78*, 3379–84.
(9) Arraez Roman, D.; Efremov, E.; Ariese, F.; Segura Carretero, A.; Gooijer, C. *Anal. Bioanal. Chem.* **2005**, *382*, 180–5.
(10) Zhang, D. M.; Xie, Y.; Deb, S. K.; Davisson, V. J.; Ben-Amotz, D. *Anal. Chem.* **2005**, *77*, 3563–3569.
(11) Faulds, K.; Barbagallo, R. P.; Keer, J. T.; Smith, W. E.; Graham, D. *Analyst* **2004**, *129*, 567–8.
(12) Cunningham, D.; Littleford, R. E.; Smith, W. E.; Lundahl, P. J.; Khan, I.; McComb, D. W.; Graham, D.; Laforest, N. *Faraday Discuss.* **2006**, *132*, 135–45.
(13) Dieringer, J. A.; Lettan, R. B.; Scheidt, K. A.; VanDuyne, R. P. *J. Am. Chem. Soc.* **2007**, *129*, 16249–16256.
(14) Nguyen, T.; Francis, M. B. *Org. Lett.* **2003**, *5*, 3245–3248.
(15) Ahern, A. M.; Garrell, R. L. *Langmuir* **1988**, *4*, 1162–1168.
(16) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850–858.
(17) Alban, A.; David, S. O.; Bjorkesten, L.; Andersson, C.; Sloge, E.; Lewis, S.; Currie, I. *Proteomics* **2003**, *3*, 36–44.

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