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Quantification of Isotope Encoded Proteins in 2-D Gels Using Surface Enhanced Resonance Raman

Giselle M. Knudsen¹, Brandon M. Davis², Shirshendu K. Deb¹, Yvette Loethen², Ravindra Gudihal¹, Pradeep Perera², Dor Ben-Amotz², and V. Jo Davisson^{1,*}

¹Department of Medicinal Chemistry & Molecular Pharmacology and the Bindley Bioscience Center at Discovery Park, Purdue University, West Lafayette, IN 47907

²Department of Chemistry, Purdue University, West Lafayette, IN 47907

Abstract

A strategy for quantification of multiple protein isoforms from a complex sample background is demonstrated, combining isotopomeric rhodamine 6G (R6G) labels and surface enhanced Raman in polyacrylamide matrix. The procedure involves isotope-encoding by lysine-labeling with (R6G) active ester reagents, isoform separation by 2-DGE, fluorescence quantification using internal standardization to water, and silver nanoparticle deposition followed by surface enhanced Raman detection. R6G sample encoding and standardization enabled the determination of total protein concentration and the distribution of specific isoforms using the combined detection approach of water-referenced fluorescence spectral imaging and ratiometric quantification. A detection limit of approximately 13.5 picomolar R6G-labeled protein was determined for the surface-enhanced Raman in a gel matrix (15-fold lower than fluorescence). High quantification accuracies for small differences in protein populations at low nanogram abundance were demonstrated for human GMP synthetase (hGMPS) either as purified protein samples in a single-point determination mode (3% relative standard deviation, RSD%) or HCT116 human cancer cellular lysate in an imaging application (with 16% RSD%). These results represent a prototype for future applications of isotopic surface enhanced resonance Raman scatter to quantification of protein distributions.

Keywords

Surface Enhanced Resonance Raman (SERRS); rhodamine 6G; quantitative 2D-gel electrophoresis; water internal standard (WIS) fluorescence; human GMP synthetase isoforms

Introduction

Despite major advances in protein analysis, accurate and precise methodologies capable of assessing changes in protein distributions in biological systems represent a target for development. Specifically, post-translational modifications of proteins on the biological response time scale requires simultaneous monitoring of multiple protein isoforms to understand signaling. Examples include signal transduction pathways controlled by phosphorylation, such as activation of MAPK cascades which is carefully tuned in terms of magnitude (1,2), timing (3), duration (4,5), and spatial regulation in response to specific stimuli (6). The relative changes in ERK1/2 isoforms can vary from 5-50% on the 10 minute time scale in Ras activation (3) to 80-90% in response to amphetamine stimulation (7), and multiple

*Address all correspondence to: V. Jo. Davisson: davisson@purdue.edu, 575 Stadium Mall Dr., West Lafayette, IN 47907, 765-494-1414 (fax). 765-494-5238 (office).

isoforms are known for signaling proteins such as src kinase (8). Small changes in protein isoform content can translate into biologically meaningful distinctions due to downstream signal amplification. A different example is enzyme isoform diversity through post-translational modifications, such as those in GAPDH which lead to variations of protein function aside from the catalytic activity (9-11).

Quantification of protein “isoforms” has been demonstrated using quantitative mass spectrometry (12) and differential gel electrophoresis (DIGE) (13). Bioconjugate chemistry combined with mass spectrometric methods have enabled a “bottom up” peptide analysis approach using stable isotope labeling of peptides *e.g.* ICAT, iTRAQ, AQUA or GIST (12). Label-free quantification methods are based on the abundance of specific ions sampled over a chromatographic peak (12,14). This method requires careful selection of survey and fragment spectra (MS and MS/MS) sampling times relative to the chromatographic peak width. Recently, a targeted approach involving the use of multiple-reaction monitoring (MRM) to track a selected precursor ion, diagnostic of a specific molecular entity, has demonstrated an improved dynamic range of 10^4 - 10^5 for phosphopeptide monitoring (12,15). However, MRM has limitations in terms of reproducibility of proteome coverage, and the relative phosphorylation status for specific protein species is not readily accessible.

Comparative proteomic strategies using 2-DGE provide an avenue for potential quantification of changes in thousands of protein isoforms provided appropriate resolution of the targeted proteins in the sample (16). In practice, only the most abundant proteins in a proteome can be recovered in reasonable amounts for further analyses, and proteins with low levels of expression remain difficult to detect, quantify and identify. This is a particularly important problem because even in typical mammalian cells, the protein copy number can vary over a 10^6 range (17), and the expected number of unique protein entities can be as high as 10^5 . This scenario is more challenging for serum proteomes (18), where the dynamic range has been estimated at up to 10^{12} .

Our efforts recently assessed the capacity of bioconjugation chemistry combined with silver-enhanced Raman detection based upon isotope ratio standardization in gel matrices to provide accurate quantification of proteins (19). Several limitations of fluorescence-based quantification may be addressed using surface enhanced resonance Raman scattering (SERRS) spectroscopy. While internal standardization of single fluorophore content in gels has been demonstrated using Raman water signals (20), the capacity to multiplex with fluorescence requires the use of multiple dye classes. These distinct chemical modifications create higher order separation problems for labeled proteins in mixtures (21). Previous applications of surface enhanced Raman-based detection for quantification have been limited due to variations in surface chemistry leading to large fluctuations in enhancement efficiency (22,23). Isotopically-encoded Raman active dyes such as Rhodamine 6-G (R6G) provided highly reproducible and quantitative SERRS-based concentration measurements (24). While Raman spectra of R6G isotopic variants are distinct, their physiochemical properties are virtually identical, thus making them ideal labels for internally standardized proteomic separations and quantification applications (19,24).

Here we present a new protein-encoding strategy which makes use of isotopic pairs of R6G-NHS ester labeling reagents for internally standardized ratiometric quantification analysis in silver stained 2D gels (19). The ratiometric quantification accuracy, gel to gel reproducibility and detection limit were assessed with a purified recombinant protein of interest, human GMP synthetase. The lower limit of detection for the SERRS measurement enabled a reduction in labeling and minimized separation artifacts. Three endogenous protein isoforms were quantified in cell lysates using internalized sample spiking with known quantities of recombinant hGMPS protein isoforms. The precision and accuracy in the resulting Raman-

based quantifications justifies future development of isotope edited internal standardized SERRS biomolecular quantification approach to protein isoform quantification methods.

Materials and Methods

Materials

Isotope encoded d₀- or d₄-R6G NHS ester reagents were prepared by S.K. Deb as reported (19). All 2-DGE materials were from Biorad. Recombinant human GMP synthetase (hGMPS) was a gift from Justin C. Oliver, produced as described to >95% purity (25). All other reagents were purchased from Sigma.

Soluble Cell Lysate Preparation

HCT116 colon cancer cells (ATCC) were cultured using standard cell culture conditions in DMEM-F12 medium containing 10% FBS. Cells were harvested by trypsin release, then rinsed three times with PBS, and pelleted for storage at -80 °C. For lysis, thawed cell pellets were suspended in chilled lysis buffer containing: 20 mM HEPES, pH 7.5, 0.25 M sucrose, 3 mM MgCl₂, 0.5% NP40, 2 mM DTT, and 1× Halt protease inhibitor cocktail (EDTA-free, Pierce). The cells (2 × 10⁷ cells/ml) were homogenized with fifteen strokes of a 2 ml Dounce homogenizer and pestle B, and then cellular debris was pelleted at 12,000 g for 20 min at 4 °C. The supernatant was concentrated and buffer exchanged into 50 mM borate buffer pH 8.5 using 5 kDa MW cutoff ultrafree spin filters (Millipore), and the protein concentration was determined using the Biorad protein assay with BSA calibration. Prepared lysates were used immediately for labeling and 2-DGE analysis.

Protein Labeling

For lysate or recombinant protein sample labeling, 100-150 µg of protein was dissolved in 50 mM borate buffer pH 8.5 to a final volume of 200-1200 µl, to which was added 1 nmol (1 µl) of d₀ or d₄-R6G-NHS reagent stock dissolved in ethanol. After 1 h incubation in the dark at room temperature, the protein reaction was desalted on a PD-10 column (GE Healthsciences) into 50 mM Tris pH 7.5 buffer. The total protein eluate was concentrated then buffer exchanged with 8M urea, 10 mM Tris pH 7.5, and 0.5 % CHAPS on a 5k MW cutoff microspin filter, to remove any remaining unreacted dye. Protein concentration was determined using the Biorad protein assay, and dye content was assessed by UV-Vis absorbance using $\epsilon = 116,000 \text{ M}^{-1}\text{cm}^{-1}$ at λ_{max} of 540 nm for R6G. These reaction conditions produce a low molar labeling efficiency (~0.1%), and were optimized using controlled mixtures of proteins with known pI and Mw and various dye/protein reaction stoichiometry, selecting for conditions that produced unaltered 2DGE images between labeled vs. unlabeled samples (20). This method minimized the appearance of charge-shifts in the IEF dimension, due to potential side-reactions with Tyr, Thr, or Ser residues (26).

2D-Page

For isoelectric focusing, protein in a volume less than 20 µl was diluted into sample rehydration buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, and 0.001% bromophenol blue for rehydration of 11 cm, 3-10 non-linear IPG strips. The IEF strips were focused under optimized conditions for mammalian cell lysates (27) over 16-20 h for a total of ~60,000 Vh using a shallow gradient over five voltage steps, reaching a maximum of 8,000V on a Biorad Protean IEF Cell apparatus. After focusing, the strips were reduced and alkylated in equilibration buffer containing 6 M urea, 0.375 M Tris pH 8.8, 4% SDS, 20% glycerol containing 2% DTT for 10 min, then 2.5% iodoacetamide for 10 min. For the second dimension, the strips were run on single well, pre-cast, Criterion 12.5% acrylamide Tris-HCl gels. The gels were fixed in 50% methanol, 5% acetic acid for 1-2 h, followed by 50% methanol

1 h, then stored in water at 4° C. Gels of non-R6G labeled control protein samples were stained with SyproRuby according to the manufacturer protocol (Biorad).

Fluorescence Scanner Gel Documentation—Fluorescence images of 2D gels were recorded using a Typhoon fluorescence scanner (GE Life Sciences), with the following settings for R6G: 532 nm excitation, 580 ($\pm$ 30) nm bandpass emission filter, 100 micron pixel size, and the photomultiplier voltage was adjusted for maximum dynamic range of signal, typically 650 Volts. For SyproRuby detection, 532 nm excitation and the 610 ($\pm$ 30) nm bandpass emission filters were used.

Silver Staining

Polyacrylamide gels were silver stained according to the Schevchenko method with modifications to minimize background staining (28,29). Due to the importance of silver substrates for SERRS detection, details of the silver staining procedure are given here. The gels were reduced for two minutes in freshly prepared 2 mM sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$). Then the gels were rinsed twice in water over 5 min before incubation in chilled (4° C) 0.1% silver nitrate solution for twenty minutes. The gels were then rinsed with two exchanges of water over 4 min, and then incubated in 10% sodium carbonate containing 0.04% formaldehyde until the protein spots had developed sufficient brown staining for visualization. Development was quenched before the appearance of excessive mirroring with a two minute wash in 5% acetic acid. Where indicated, gels were preserved by soaking in 10% glycerol and drying between sheets of cellophane at room temperature.

d_0/d_4 -R6G Ratiometric Accuracy

The practical limits of the ratiometric quantification strategy were assessed using mixtures of separately labeled d_0 - or d_4 -R6G-recombinant hGMPS. Gel-to-gel reproducibility and precision were assessed for a 43% d_4 -R6G composition sample, representing a 1.3-fold difference between d_0 - and d_4 -R6G concentrations. Three replicate gels containing 3 μg total of the mixed protein sample were separated by 2-DGE, silver stained and then dried as described above. A practical detection limit was determined by sampling a series of 2-DGE samples containing 3 μg , 300 ng, 30 ng or 3 ng total hGMPS protein. Here the sample composition was selected to be 64.2% d_0 -R6G, representing a 1.8-fold difference between d_0 - and d_4 -R6G samples, a value selected to be significant at picomolar R6G concentrations. For these samples, a single-point determination procedure was followed using a Senterra confocal Raman microscope (Bruker Optics) operated with Opus v. 6 software.

The Senterra was equipped with a 532 nm laser, and Olympus MPlan 20 $\times$ (0.4 NA) or 50 $\times$ (0.75 NA) objectives, with collection spot size dimensions of 50 $\times$ 1000 μm , and 3-5 cm^{-1} resolution grating settings. The following settings were used to acquire spectra for 0.1-1 μg protein: 2 mW power, 2-5 scans, 30 second integration time, and for 1-10 ng protein: 5-10 mW, 5 scans, and 60 second integration. Data were fit using a manual curve fitting operation within Opus software around the signature 610 and 600 cm^{-1} peaks, using baseline subtraction between 619 and 587 cm^{-1} , and a Gaussian peak shape model. The ratio of areas of the 610 to 600 cm^{-1} peaks directly provided values of d_0/d_4 composition.

d_0/d_4 -R6G Labeled Spiked Lysate

Preparation—To produce spiked lysate samples, HCT116 cell lysate was divided equally into three vials containing 750 μg lysate, to which was added 0, 1.5 or 3 μg of recombinant hGMPS. These samples were labeled in separate reactions to give two d_0 -lysates containing w/w 0% or 0.2% spiked recombinant hGMPS and a single d_4 -lysate containing 0.4% spiked recombinant hGMPS. Mixed composition d_0/d_4 samples were combined as follows: *Sample A*: 100 μg each of the d_0 -labeled, 0% spiked and d_4 -labeled, 0.4% recombinant hGMPS-spiked

lysates, *Sample B*: 100 μ g each of the d₀-labeled, 0.2% spiked and d₄-labeled, 0.4% spiked lysates. The d₀-R6G labeled, non-spiked lysate sample was also used as a control (*Sample C*).

Theoretical Percent d₄-R6G Composition in Spiked Lysates—The theoretical percent d₄-R6G composition in a given hGMPS protein spot was calculated as a function of the composition of endogenous hGMPS and the recombinant protein that was added to it. For each protein spot, endogenous (E) protein was defined as the mass (ng) of hGMPS present in 200 μ g of cell-free lysate. The mass of recombinant (R) protein (ng) in a protein spot was calculated per 200 ng recombinant protein (or 0.1% w/w addition of recombinant protein to 200 μ g of lysate). The theoretical composition of d₄-R6G (%) in Samples A and B was then given by equations 1 and 2.

$$\%d_4(\text{sample A}) = \frac{(\frac{E}{2} + 2R)}{(E + 2R)} \quad (1)$$

$$\%d_4(\text{sample B}) = \frac{(\frac{E}{2} + 2R)}{(E + 3R)} \quad (2)$$

Multispectral Fluorescence and Raman Imaging

Full-spectral imaging was performed using a custom built micro-Raman system (30), equipped with an air-cooled 514.5 nm Ar⁺ laser (Melles-Griot) and an Olympus BX41 microscope. Raman scatter was collected using a 20 $\times$ objective (Olympus ULWD MSPlan, 0.4 NA) and focused onto a circular-to-linear fiber bundle for detection using an imaging spectrograph (Acton Research, SpectraPro 300i) and a 1024 $\times$ 256 LN-cooled CCD (Princeton Instruments). Gel imaging was performed using a custom written LabVIEWTM (National Instruments) program to raster scan the gel through the laser focus while collecting spectra from an array of points in the gel. The laser focal spot size was 10 μ m in diameter and 100 μ m in depth, effectively 10 pL focal volume, and the x-y step size in fluorescence or Raman scatter images was 200 or 250 μ m. Gels were placed on a low-fluorescence glass plate (Bio-Rad) under a small pool of Milli-Q water to prevent drying over the duration of the scan. Fluorescence imaging parameters were: laser power, 1 mW at the sample; spectrograph grating, 300BLZ at 670 nm; exposure time, 5 msec; image size, $\sim 70 \times 40$ pixels = 17.5 $\times$ 10 mm; scan time = 10 min. SERRS imaging parameters were: laser power, 14 mW at sample; spectrograph grating, 1200BLZ at 554 nm; exposure time, 1 sec; image size, $\sim 48 \times 48$ pixels = 12 $\times$ 12 mm; scan time = 1 hour.

Analysis of Multispectral Fluorescence and Raman Imaging

Absolute Dye Quantification by Fluorescence—For absolute R6G quantification in protein spots, the water-Raman subtraction method (WIS) of Loethen *et al.* (20) was applied to a small region of the gel containing spiked sample. Calibration of dye concentration in solution was performed using d₀-R6G (calibration plot shown in Supplementary Figure 1), and an additional region was imaged from the gel margin (protein-free) for calculating an average spectrum to be used as background. Savitzky-Golay second derivative solvent subtraction analysis of all spectra yielded the normalized value of R6G fluorescence to the water Raman band (31,32). Dye concentration was calculated over the volume of a protein spot using the protein-free R6G calibration and the normalized fluorescence spectral integration obtained from each image pixel. Using this method, a user-defined protein spot was selected as an

internal standard for which the absolute molar amount of dye was quantified. Thus the relative fluorescence in this spot could be used to normalize signal across replicate fluorescence gel images.

Automated SERRS Spectral Processing—To remove the fluorescence background from SERRS spectra, an automated method of polynomial fitting was used to model a composite fluorescence spectrum that could be subtracted (33). This procedure involved pixel by pixel comparison of the original spectra with a fourth-order polynomial fit, and generating a composite spectrum by inputting the lesser of the original spectrum pixel value or the polynomial pixel value. Five or more iterations were performed to arrive at a composite spectrum that contained little or no Raman bands, smoothed using a second-order Savitsky-Golay width of 25 pixels (32), and subtracted from the original spectrum yielding a near null-baseline SERRS spectrum.

Partial least squares (PLS) modeling was used to quantify d₀ and d₄-R6G composition, using full-range, baseline-subtracted, second derivative SERRS spectra. To generate the PLS model, a training set was collected with 200 spectra obtained for each of the following % d₄-R6G compositions 100%, 75%, 50%, 25%, or 0% of a standard, d₀- or d₄-R6G-labeled purified protein (ferritin) (19). These spectra were weighted by standard deviation over the 200 spectra for each set, but otherwise were not manipulated for this analysis. The final calibration plots for PLS modeled training set data with observed versus expected %d₄-R6G composition had a linear R² correlation coefficient of 0.999 (Supplementary Figure 2).

Results

Quantitative Fluorescence and Raman Imaging

An analysis of cellular lysates as typical complex proteomic samples were used to establish the working protein and R6G concentrations for the overall quantitative fluorescence and Raman imaging strategy. Sample labeling used either 1A or 1B (Figure 1) (19) followed by 2-DGE separation, fluorescence spectral imaging, silver nanoparticle deposition, and Raman spectral imaging (Figure 2). In Figure 3 is shown the 2-DGE analysis of 30 µg soluble lysate sample prepared from HCT116 human colon cancer cells. This sample was labeled using R6G-NHS ester lysine labeling reagent 1A (Figure 1), at a ratio of 6.6 picomoles dye per microgram protein to yield on average ~0.1% labeling efficiency. Throughout this study, lower labeling levels were selected to minimize potential electrophoretic mobility shift effects (34). This reaction stoichiometry is reduced relative to typical sample labeling protocols (35), which yield approximately 1-2% protein labeling efficiency (21).

The absolute dye content in a single protein spot on the 2D gel was established using a water internal standardization method that compares the fluorescence of R6G to the water Raman signal (20). The corrected spectra for the R6G fluorescence intensity image is shown in Figure 3B, with sample spectra from the designated protein spot shown in Panel 3C. The concentration of R6G in this protein spot was determined to be 3.1 nM, averaged over the gel volume with dimensions as drawn in Panel 3B. The detection limit was defined as 3× noise and routinely set at 0.2 nM. The Raman intensity image for this gel region (panel 3D) accurately represented the fluorescence image (panel 3B), and the Raman spectrum shown in Panel 3E was obtained from the selected protein spot containing 3.1 nM R6G.

Highlighted in these results is the use of a standard silver stain for acrylamide protein gels to generate a viable SERRS substrate formed *in situ*. Cryo EM imaging has shown that the sizes of brown, yellow, or blue-grey silver nanoparticles obtained in gels using silver deposition are typically 20-100 nm in size; these nanoparticles are within the range expected for surface enhanced Raman substrates (36,37). These sizes correlate well with data from size-controlled

preparations of silver aggregates often used for surface enhanced Raman (38,39). Raman enhancement factors or efficacy were not analyzed and no attempts to optimize the nanoparticle preparations (in terms of nanoparticle sizes, geometries, and roughness) were pursued.

GMP Synthetase Isoforms

For purposes of testing the feasibility of isotope edited internal standardization SERRS for protein isoform quantification, recombinant human GMP synthetase (hGMPS) posed a useful model protein. Human GMPS is encoded by a single gene (on the 3q chromosome) and only a single mRNA form has been reported, yet it has been described in at least two distinct isoforms with different isoelectric points, as isolated from the T-lymphoma A3.01 cell line or produced in a recombinant baculovirus expression system (40,41). A differential phospho-peptide analysis of the human tumor-derived cell line A431 indicated that Thr330 of hGMPS undergoes a phosphorylation event (42). Understanding which isoform is linked to a particular hGMPS function is an emerging question; an example being the elevated expression in tamoxifen-resistant xenograft breast tumor 3366 cell line (43). The recombinant protein is purified as multiple species termed here as “isoforms”, that resembled those found in endogenous HCT116 cell lysate as shown after 2-DGE separation (Figure 4). By fluorescence densitometry quantification using SyproRuby staining, the hGMPS protein species identified as E1-E4 in Figure 4B (MS/MS data in Supplementary Table 1) composed 0.1-0.2% of the total 200 µg protein lysate, or 200-400 ng, in the gel. Four species were also identified in recombinant hGMPS purified from an *E. coli* expression system (labeled R1-R4 in Figure 4C, MS/MS data in Supplementary Table 2), suggesting that the modification of these putative hGMPS isoforms could originate from a similar chemical or biochemical process in both samples.

Gel-to-Gel Reproducibility of Ratiometric Quantification

An experiment was conducted to assess gel-to-gel reproducibility of the Raman spectral analyses using d₀- and d₄-R6G-labeled recombinant hGMPS at a fixed %d₄-R6G composition of 43%. Three replicate silver stained gels were prepared containing approximately 3 µg of total recombinant protein (or approximately 1 µg per protein species), and six Raman spectra were recorded for the most abundant hGMPS species (Figure 5). These spectra were submitted to Gaussian curve fitting analysis of the signature 611 and 600 cm⁻¹ peaks to obtain d₀- and d₄-R6G composition directly; %d₄-R6G composition in each of the three gels (averaged over six spectra) was: 43.3 ± 1.8%, 42.6 ± 0.6%, and 42.9 ± 1.3%. Percent relative standard deviation (RSD%) was used to summarize overall variance for the %d₄-R6G compositions, as described recently by Schröder *et al.* (44). Using single gel measurements (with *n* = 6 spectra), the RSD % for the three individual gels was 4.3%, 1.8%, and 3.1%. The value gained in recording data from three gels versus one gel was assessed by combining all eighteen spectra from the three gels, resulting in an RSD% of 3.0% overall. Therefore, there was no great advantage gained from recording SERRS spectra from multiple gels in this analysis, avoiding various sources of reproducibility error in 2-DGE (44). The result highlights the inherent accuracy in the ability of Raman to simultaneously detect isotopomeric dye populations in the same spectra.

Detection Limit of Ratiometric Quantification

To assess the practical detection limit of the ratiometric measurement, recombinant hGMPS samples were prepared in a ten-fold dilution series and developed on 2-DGE. In these samples, 64.2% d₄-R6G composition (or 1.8-fold difference between d₀- and d₄-R6G) was chosen to allow for absolute quantification errors over the range of nM to pM concentrations. The expected concentration of dye in the ~1 ng protein per spot sample was estimated as 75 pM based on 0.1% labeling efficiency, 76 kDa molecular weight, and approximate spot size of 0.5 × 0.5 mm². The most abundant protein species were quantified in each of these gels and found to contain %d₄-R6G of: 63.9 ± 1.0%, 64.5 ± 1.2%, and 64.3 ± 2.5%, for the 1 µg, 100 ng, and

10 ng per spot gels respectively. Signal to noise ($S/N = 2$), acquisition time (~ 30 min), and background became limiting in the 1 ng gel for this simple Gaussian peak fitting method, and only three spectra could be manually fit for this sample. If, however, the peak shape model from the 1 μ g-10 ng samples was applied to the 1 ng sample spectra, a %d₄-R6G of $64.9 \pm 6.1\%$ could be obtained (over $n = 3$ spectra). Thus the practical protein detection limit was approximately 1 ng per spot for a simple single-point measurement method, comparable to silver stain (0.3-10 ng/spot), Sypro Ruby dye (1-2 ng), and Cyanine dye covalent labeling (0.25-0.95 ng) protein detection limits in gels (45,46). Using the metric of three-fold variance in the 75 pM sample, the SERRS detection limit in terms of dye concentration was on the order of 13.5 pM.

Ratiometric Quantification of hGMPS Isoforms in Lysate

To evaluate the ratiometric quantification strategy in typical proteomic samples, an imaging-based internally standardized analysis was designed to quantify small-fold changes in hGMPS isoform distribution with a constant protein background in a complex cell lysate. In this experiment, two pair-wise ratiometric analyses were performed with recombinant hGMPS spiked lysates. The tasks were to determine both the accuracy in %d₄-R6G composition for multiple hGMPS species and the composition of endogenous hGMPS in the lysate.

SERRS imaging was used for direct quantification of d₄-R6G composition in mixed lysate samples containing 0, 0.2 or 0.4% mass spiked hGMPS, analyzed as Samples A and B. The results reported in Table 1 were based upon single gel analyses for Samples A and B (Figure 6), as justified by the gel-to-gel reproducibility experiment. Each pixel in Figure 6 represents a full R6G Raman (SERRS) spectrum.

Representative SERRS spectra that compose the image maps of Samples A and B are shown in Figure 6, and the complete dataset is provided in Supplemental Figure 3. These spectra ($48 \times 48 = 2304$ per image) were fitted using a partial least squares analysis that had been modeled using a training set of 1000 spectra recorded at controlled %d₄-R6G compositions (described in the experimental section). The percent d₄-R6G compositions in each of the hGMPS and background species (Table 1) were calculated by PLS analysis and averaged over three spectra per protein spot.

As a measure of overall variance for this ratiometric quantification imaging method, the total RSD% was determined separately for Samples A and B, using the average and standard deviations of replicate readings for the eight identified protein species reported in Table 1. For both Samples A and B, the total RSD% is 16%. Using the high scan rate, low signal-to-noise Raman spectra, the quantification strategy had a total RSD% that was at least comparable with fluorescence-based methods evaluated at similar protein concentrations. For example, the total RSD% from native protein fluorescence detection was reported to be 12-16% (44), and data reported in a benchmarking study for the DIGE method had a total RSD% of $\sim 31\%$ for four standard proteins analyzed with pooled sample internal standardization. By way of comparison, a recent report using the SILAC quantitative MS method, which also employs isotope encoding (47), had an overall RSD% of 11% within a narrow 3-fold difference range. The Raman spectra used here were acquired using settings optimized for speed in an imaging application, but which resulted in relatively high noise levels and is the primary source of variance in these measurements.

hGMPS Isoform Quantification in HCT116 lysate

The mass composition of endogenous hGMPS could be determined in the four putative isoforms of hGMPS (species 1-4) by defining the recombinant hGMPS sample as the internal standard. Recombinant hGMPS (R) was quantified independently by fluorescence

densitometry analysis of the 2-DGE analysis shown in Figure 4C and with knowledge of the protein concentration. Given the Raman measured %d₄-R6G compositions reported in Table 1, it was possible to solve for the endogenous hGMPS (*E*) values reported in Table 2 using equations 1 and 2. The %d₄-R6G compositions in Samples A and B were determined from independent silver-enhanced Raman experiments; therefore it is significant that the calculated endogenous protein values were consistent for these two experiments. For the least abundant hGMPS species (species 4), the endogenous amount could not be determined due to the low-abundance in the recombinant internal standard. It was apparent that the mass distribution among endogenous isoforms of hGMPS, with the most abundant species E2 (Panel 4B) was pI-shifted to a more acidic form relative to the major recombinant species (R1 in Panel 4C). One interpretation would be that the most basic recombinant hGMPS species (R1) was an unmodified or native parent species, while the remaining putative hGMPS isoforms (2-4) were likely to be modified versions of this protein found in the endogenous sample.

Discussion

The potential for application of isotope encoded internally standardized SERRS detection to quantification of multiple protein isoforms within a complex biological sample background is now apparent. R6G-protein encoding has enabled the absolute quantification of sample in hydrated gels using water-internal standardized fluorescence spectral imaging. The particular combination of chemical features in d₀- and d₄-R6G labeling reagents, and *in situ* silver nanoparticle formation around protein in a gel matrix has enabled highly accurate relative quantification using SERRS detection with an established protein separation workflow. SERRS occurs when a molecular species occupies specific “hot spots” on metal surfaces such as silver, gold or copper, with curvature features that are smaller than the wavelength of the incident light (48). Local electric field enhancement by metal surface plasmons lead to enhanced Raman scattering from adsorbed molecular species, by a factor of 10⁶ or more. Furthermore, under electronic resonance (SERRS) conditions, enhancements on the order of 10¹⁴ can be obtained, thus allowing single molecule detection (49,50). Isotope editing provides for the first time a solution to a recurring problem with variations in SERRS efficiencies (51, 52), enabling application of Raman features to quantitative biomolecular detection.

The inherent accuracy of isotope encoded internally standardized SERRS ratiometric quantification was demonstrated in analyses of gel-to-gel reproducibility and practical detection limits for the method. Sample to sample variations in gel efficiency (44) are eliminated through the use of internal standardization, as was demonstrated by comparison of error between single and multiple gel analyses. Furthermore, the ability to simultaneously monitor two isotopomeric species of R6G in a single SERRS spectral measurement allows for significant reduction of Poisson noise in the measurement due to variations in SERRS efficiency, therefore expanding the practical detection limit into the low picomolar dye concentration realm. For example, in the 10 ng per spot samples, the d₀ and d₄ peak area results obtained from five replicate measurements were 427 ± 260 and 746 ± 377 counts, respectively. If the variance in the peak areas were uncorrelated (Poisson noise) then it would not be possible to determine the value of the area ratio with an accuracy of better than about ± 50%. The fact that the experimentally measured standard deviation of the isotopic area ratios obtained from these same samples was ± 2.5% indicates that most of the variance in the peak areas is highly correlated. In other words, because the d₀ and d₄-R6G chromophores are virtually chemically identical, the areas of the resulting SERRS peaks vary in unison, in spite of the large batch to batch variations in the SERRS activity of the sample.

Based on throughput and protein identification, mass spectrometry is often preferred over optical detection methods for quantitative proteomic analysis. However, it is arguable that the identity of a protein spot on a 2-D gel only needs to be determined once when studying the

dynamics of the species in a known system. We have shown that it is possible to accurately determine relative compositions of isotopically labeled protein samples at low nanogram protein levels with the caveat that species identification and 2-D gel position must be proven at higher concentrations, since the SERRS detection limit is effectively 15-fold lower than fluorescence.

In this work, we have demonstrated accurate and precise quantification of three major species of human GMP synthetase through internal standardization. In a single point determination mode, purified recombinant hGMPS species could be quantified in single gels with a 1-6% error over a 1 μ g to 1 ng protein mass range. In an imaging application using automated spectral analysis, an RSD% of 16% could be obtained. Future applications will likely combine imaging and single-point determinations for high accuracy quantification. This level of detection, accuracy, and precision in protein isoform quantification represents a new benchmark for isoform quantification (13,44,47), and sets the stage for future applications of isotopic surface enhanced Raman to quantitative analysis of biomolecules.

Conclusion

In this study we have demonstrated a new application of SERRS detection to the quantification of protein distributions, through the use of isotopically paired R6G lysine labeling reagents for sample encoding. By virtue of the inherent accuracy of Raman-based spectral quantification of isotopic dye populations, this strategy is uniquely positioned to overcome both the challenges of 2-DGE due to the reproducibility as well as variations in SERRS efficiency through ratiometric analyses. Furthermore, there is a strong implication in the matching of the detection capacity of SERRS limit of detection and dynamic range to the scale of protein abundances found in proteomic samples. In particular, an approach to accurately assess the graded variance in individual protein species has been established. This work has shown that the ratiometric comparisons that can be made by using isotopic variants of SERRS-active dyes as internal references results in highly accurate and precise quantification of protein distributions, and justifies the future development of SERRS-based biomolecular quantification using isotope encoded internal standardization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of d₀- and d₄-R6G-NHS ester labeling reagents (1A and 1B); asterisks denote the sites of deuterium incorporation and the site of nucleophilic attack by lysine residues for protein labeling is indicated.

Figure 2.
Sample handling workflow.

Figure 3.

Shown here is a 2-DGE analysis of 30 μg of R6G-labeled lysate. (A) The full-sized fluorescence image was recorded with a Typhoon fluorescence scanner. (B) The 13 $\times$ 13 mm inset region was further imaged using our confocal system by fluorescence (200 μm step size). (C) Representative fluorescence spectra are shown for protein (on the $\times 10^3$ scale), background (on the $1 \times$ scale), and water-subtracted background ($1 \times$ scale). (D) The gel was silver stained, then imaged by Raman in the same inset region, color coded for intensity at 1650 cm^{-1} from red (high) to blue (low). (E) A representative background-subtracted SERRS spectrum from the marked protein spot is shown.

Figure 4.

Shown here is a comparison between endogenous and recombinant hGMPS protein species in unlabeled, SyproRuby stained 2-D gels. **(A)** The full scale fluorescence image of 200 µg of HCT116 lysate was recorded using a Typhoon scanner. **(B)** The inset region of panel A was enlarged and contrasted to show putative endogenous species of hGMPS, labeled E1-E4. **(C)** The 2-DGE image of 1 µg of recombinant hGMPS is shown on the same scale as panel B and contained four distinct species of hGMPS (R1-R4).

Figure 5.

Baseline corrected SERRS spectrum of d_0/d_4 -R6G labeled recombinant hGMPS in a 2D gel. The inset spectrum shows an expanded view of the 600 cm^{-1} spectral region containing the well resolved d_0 (right) and d_4 (left) peaks.

Figure 6.

Raman mapping images are shown for Samples A and B, in panels **A** and **B** respectively. Protein species labeled 1-4 in Samples A and B were confirmed to be hGMPS by in gel trypsin digestion and MS/MS peptide sequencing (Supplemental Table 3). Also shown are the background lysate proteins, labeled b1-b4, selected for %d₄-R6G composition calculations. Scale bar represents 2.5 mm. Representative baseline corrected SERRS spectra from Samples A and B are shown in panels **C** and **D** respectively. Signal from the background protein species b1 (solid trace) is compared with signal from the hGMPS species 1 (dashed trace). The inset shows the signature d₀-R6G (611 cm⁻¹) and d₄-R6G (600 cm⁻¹) peaks, with d₄-R6G signal enrichment in the dashed hGMPS species traces as expected in these spiked samples. The full set of SERRS spectra from hGMPS and background species in Samples A and B is provided in Supplemental Figure 3.

Table 1

Percent d₄-R6G composition in hGMPS and background protein species in Samples A and B, determined by full-spectral Raman analysis.

Protein Species	%d ₄ (Sample A)	%d ₄ (Sample B)
1	86 ± 4	66 ± 5
2	76 ± 8	60 ± 5
3	70 ± 2	56 ± 9
4	50 ± 9	49 ± 9
b1	46 ± 8	45 ± 3
b2	43 ± 8	52 ± 8
b3	40 ± 13	46 ± 15
b4	46 ± 1	57 ± 5

Table 2

Quantification of endogenous isoforms of hGMPS species (*E*) in Samples A and B, as directly determined from SERRS spectra and using recombinant protein (*R*) as an internal standard.

hGMPS species	Recombinant [□] (<i>R</i>) (ng/100 ng)	Endogenous (<i>E</i> _(Sample A)) (ng/100 µg lysate)	Endogenous (<i>E</i> _(Sample B)) (ng/100 µg lysate)
1	46.2	35.2 ± 2.5	34.6 ± 3.8
2	36.5	66.2 ± 9.6	65.5 ± 7.1
3	12.6	38.1 ± 1.5	37.2 ± 8.1
4	0.6	<i>nc</i> [*]	<i>nc</i>

[□] Recombinant protein distribution was normalized to 100 ng, equivalent to 0.1% w/w in 100 µg lysate.

^{*} The value of endogenous protein species 4 could not be calculated (*nc*).