World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:6, No:3, 2012

Quantification of Peptides based on Isotope Dilution Surface Enhanced Raman Scattering

F. Yaghobian, R. Stosch, and B. Güttler

***Abstract*—**This study aims to demonstrate the quantification of peptides based on isotope dilution surface enhanced Raman scattering (IDSERS). SERS spectra of phenylalanine (Phe), leucine (Leu) and two peptide sequences TGQIFK (T13) and YSFLQNPQTSLCFSESIPTPSNR (T6) as part of the 22-kDa human growth hormone (hGH) were obtained on Ag-nanoparticle covered substrates. On the basis of the dominant Phe and Leu vibrational modes, precise partial least squares (PLS) prediction models were built enabling the determination of unknown T13 and T6 concentrations. Detection of hGH in its physiological concentration in order to investigate the possibility of protein quantification has been achieved.

***Keywords*—**Surface Enhanced Raman Scattering, Quantification, Peptides.

I. Introduction

THE characterization and quantification of protein biomarkers in human serum has the potential for biomedical analysis in early diagnosis and monitoring of specific diseases like cancer, AIDS or Alzheimer [1-11]. To ensure the reliability of clinical laboratory measurements for their diagnoses and to provide traceability of the results to the relevant SI units (mole, kg), routine test procedures have to be linked to higher order standards. Amount of substances in each of these measurements is the focus of being traceable. Hence, the quantification of proteins at a primary level is still a challenging and on-going topic in chemical metrology. Methodologies for SI-traceable protein quantification have been implemented recently [12,13]. The principle in this method is that particular signature peptides of the protein are obtained as cleavage products from enzymatic proteolysis and qualified by mass spectrometry (MS) in place of the protein as a whole. The required degree of accuracy and precision is achieved by application of the isotope dilution principle using isotopically labeled analogues of the probed peptides as internal standard [13].

Since the concentration of a specific protein biomarker in biological samples is typically sub-nanomolar, a highly sensitive method such as surface-enhanced Raman scattering (SERS) is used to provide the sensitivity for these types of assays. SERS is a possible alternative optical method for MS that can be applied in order to combine highest sensitivity and molecular specificity.

Physikalisch-Technische Bundesanstalt, Bundesallee 100, 38116 Braunschweig, Germany
*Dr. Fatemeh Yaghobian (e-mail: b_yaghobian@hotmail.com).

Novel metal nanostructures as SERS active substrates partially quench the fluorescence emission of biologic materials and also amplify the intensity needed for their detection in low concentrations [14-16].

Therefore detection and amplification of a broad variety of biomolecules from small to larger ones like peptides and proteins by SERS has been extensively reported [17-23]. We have already shown that the ID principle can also be adapted to SERS that increases accuracy and precision of quantitative SERS results. This has led to the new IDSERS approach which has been applied as a possible optical alternative to MS for the quantification of small diagnostic marker molecules like creatinine [24] in colloid solutions at its relevant serum concentration level. Investigating other aspects of application of novel metal nanostructures, full procedure of IDSERS based quantification has been realized as on-chip assay on suitable nanostructured solid substrates. In such an approach the detection of very low concentration (ppm-ppb), and relatively small amounts of sample material (pg) is possible from sample volume with small quantity of samples (<0.1 µl) on a single chip [16, 25, 26].

This study demonstrates a possible reference procedure for traceable quantification of peptides and even full proteins that can be developed based on IDSERS. The scope of the on-chip procedure, reported recently for small biomarkers like urea and creatinine [25, 26], is extended towards the quantification of protein biomarkers. As an example, the human growth hormone (hGH) has been investigated and models for its quantification have been developed on the bases of several characteristic of peptide sequences. Growth hormone is a 191-amino acid peptide hormone secreted by the anterior pituitary gland in the brain and enhances tissue growth by stimulating protein formation. The physiological concentration of hGH hormone containing in human serum is 60-180 pg/mL [27]. Identification is typically achieved by characteristic vibrational fingerprint of particular amino acids. Raman features of proteins are typically dominated by the vibrational modes involving the amide backbone and the aromatic amino acid side chains [28], so it is worthy to identify the spectral characteristics of aromatic amino acid residues and also their behavior in peptides in order to understand and interpret the SERS spectra of proteins.

II. Experimental Section

A. Chemicals and Reagents

Silver nitrate (Aldrich) and methanol (Merck) were of analytical grade. Ultra pure Milli-Q water (Millipore) was used

for the preparation of aqueous solutions. Recombinant 22 kDa human growth hormone (hGH) was from ProSpec-Tany TechnoGene (Rehovot, Israel). The peptide fragments T13 (TGQI(F)K, amino acids 135-140) and T6 (YSFLQNPQTS(L)CFSESIPTPSNR, amino acids 42-64) were custom synthesized and obtained from Cambridge Research Biochemicals (T13) and Thermo Electron (T6). The isotopically labeled forms are referred to as *T13 and *T6 and contain $^{13}C_9$,^{15}N-phenylalanine in position (Phe) and $^{13}C_6$,^{15}N-leucine in position (Leu), respectively. Natural L-phenylalanine was obtained from Fluka (> 99%), isotopically labeled $^{13}C_9$,^{15}N-phenylalanine was obtained from Cambridge Isotope Laboratories (98% ^{13}C, 98% ^{15}N).

Three series of each 11 reference mixtures for the systems Phe/*Phe, T13/*T13 and T6/*T6 were prepared gravimetrically. They cover the entire range of possible molar compositions between 0:100 and 100:0 and contain a constant total analyte concentration of 100 nmol/L.

Figure 1 shows the molecular structures of phenylalanine, leucine, T13 in which Phe has been labeled, T6 with the labeled leucine and hGH containing two peptides T6 and T13.

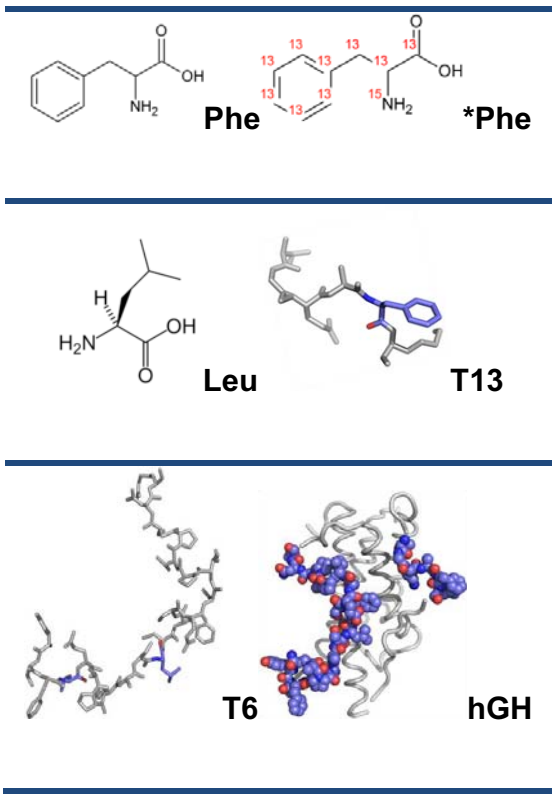

Fig. 1 Structure formulas of natural and $^{13}C_9$ ^{15}N-phenylalanine, natural leucine and stick-representations of the T6 and T13 structures with the phenylalanine and leucine entities highlighted in color and also hGH with T6 and T13

B. Nanoparticles Prepared by Electroless Deposition

Spherical silver nanoparticles were fabricated by electroless deposition. A 3-inch Si(100) wafer was coated with a 70 nm Germanium layer. Then, the wafer was cut into 5 x 8 mm rectangular pieces that were immersed for 10 min in a 1 mmol/l AgNO3 solution to induce silver nanoparticle formation. To stop the surface reaction, the substrates were first immersed in distilled water and then stored in a 1:1 (v/v) methanol-water mixture until further use. As a result, a SERS active substrate covered with spherical and nearly mono disperse silver nanoparticles of ~50 nm is obtained [25,26].

C. Sample Preparation

The procedure is schematically shown in figure 2. Several spots containing sample amounts of approximately 1 pmol are applied by pipetting an aqueous solution (0.1 µl) onto the SERS-active area of the substrate. After evaporation of the solvent, the analyte remains as thin layers forming patches of 500 µm diameter which were measured one by one.

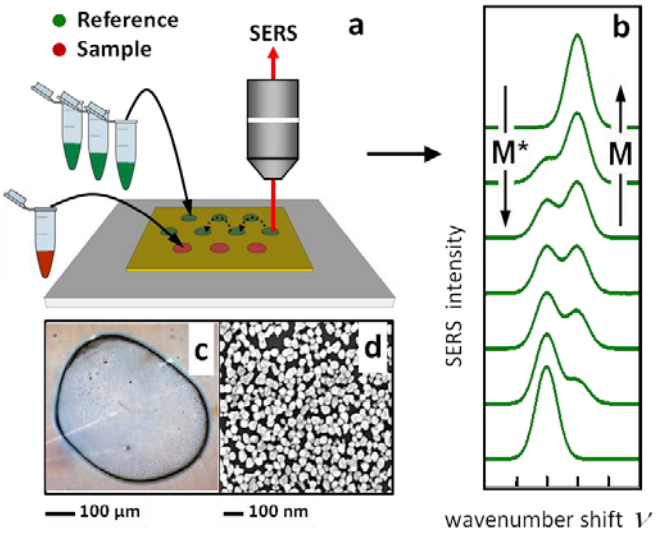

Fig. 2 (a) on-chip approach, (b) continuous transition of isotopologues, (c) SEM of AgNPs, (d) appearance and size of patches

D. Raman Instrumentation

SERS spectra were recorded using a LabRam ARAMIS Raman-spectrometer (Horiba Jobin Yvon) equipped with four holographic gratings (600, 1200, 1800 and 2400 grooves/mm) and a CCD detector thermoelectrically cooled to -70 °C. A 785 nm diode laser (100 mW) was used for excitation by focussing the beam onto the sample surface through a 100x objective resulting in a cross section of ~1 µm. The 520.5 cm^{-1} Si optical phonon mode caused by the underlying substrate was used for Raman shift calibration. Measurements were recorded at various positions inside the sample spots with integration times of 10 seconds and averaged over 30 accumulations. Spectrum acquisition and preprocessing were done with LabSpec 5.0 software (Horiba Jobin Yvon). The data was then converted to ASCII format and imported into "The Unscrambler" (CAMO Software), which has been used for generation and validation of the partial least squares (PLS) model.

III. Results and Discussion

In this work, label free detection of the 22-kDa human growth hormone (hGH) at its physiologically serum concentration, has been investigated and the SERS spectrum is shown in figure 3.

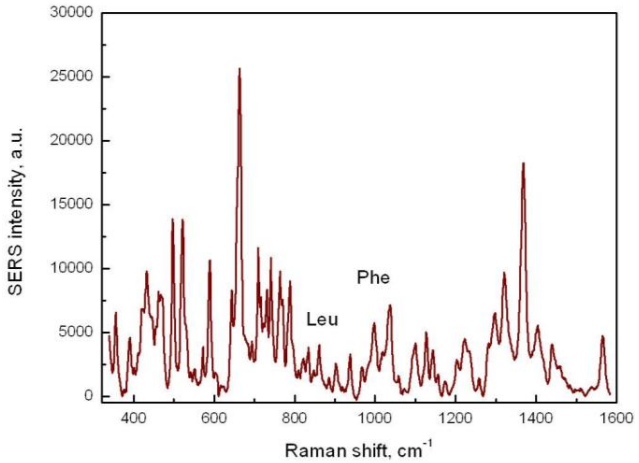

Fig. 3 SERS of hGH in its physiologically concentration obtained on substrates covered with silver nanoparticles

Quantification of peptides and proteins by means of IDSERS requires that at least one prominent Raman band could be detected which represent the peptide of interest and a sufficient wavenumber shift could be measured for the labeled isotopologue with respect to the unlabeled isotopologue. Both requirements are met here by selecting two sequences in hGH, the amino acids phenylalanine and leucine as two labeled entities (figure 4).

Together with protein backbone groups, aromatic amino acid residues provide dominant features in SERS spectra of peptides and proteins when present. It follows that the Raman modes of phenylalanine may likely apply to the assignment of Raman and SERS features in the spectra of other peptides and proteins. Since aromatic amino acids are found amongst the dominant features in the Raman spectra of peptides and proteins, it should be possible to predict at least some of the major features of the SERS spectrum of more complex proteins and peptides using the spectra of the aromatic peptides as a conceptual basis set [10]. Such behaviors have been applied in view of realizing ID based quantification.

SERS spectra of phenylalanine (Phe), leucine (Leu) and two peptide sequences T13 and T6 as part of hGH were obtained on Ag-nanoparticle covered substrates. The spectra of Phe and T13 are shown in figure 4a together with spectra of the isotopically labeled *Phe and *T13. It can be seen that the spectrum of the pure phenylalanine (Phe) is dominated by the symmetric ring stretching mode at 1003 cm^{-1} [10] which is shifted to 965 cm^{-1} due to the 10 fold isotopic labeling in phenylalanine's backbone. The isotopic shift of 38 cm^{-1} results in two fully separated bands. The peak at 1003 cm^{-1} also appears at the same position as one of the most prominent SERS modes in the spectra of the pure T13.

In labeled T6, the 7 fold isotopic labeling has been done on leucine by comparing the results for L/*L to T6/*T6, it can be seen that leucine peak dominates the spectrum at 838 cm^{-1} and actually is the amino acid responsible for isotopic shift (figure 4b). The extent of the isotopic shift in leucine (labeled/ unlabeled) is of 22 cm^{-1} and remains constant. The interesting point observed is that in SERS of leucine we can detect a relatively broad peak at 838 cm^{-1} but in labeled leucine not only the shifted mode due to the labeling is seen but also one more neighboring peak which seems to have been overlapped in the dominant peak of the leucine is observable. This peak is not shifted because it has not been a matter of labeling of carbon and nitrogen. Since the leucine which is labeled is internally connected to other amino acids in the long chain of peptides, it can be interpreted that the second neighboring peak cannot be seen in the spectrum of labeled T6.

The amino acids and peptides are bound to the surface of Ag nanoparticles via side binds or come very close to it and SERS spectra were obtained. The small amounts of available sample material require the quantification to be realized as an on-chip approach described recently [25]. The measured SERS spectra were limited to the most significant spectral range between 950 and 1030 cm^{-1} for T13 and 800 to 850 cm^{-1} for T6 which particularly reflects the continuous transition between analyte and standard, and converted into a calibration set. The selected wavenumber range particularly reflects the continuous transition between each of the isotopologues' contributions according to their proportions in the mixtures. The measurements for validations have been done with concentration of 100 nmol/L.

Two series of Phe/*Phe and T13/*T13 mixtures have been measured here and the transition from T13 to *T13 in 11 steps is shown in figure 4b. The experiments have been done for the mixture of T6 and *T6 as well. The obtained result of T6 as a longer peptide and *T6 where leucine is labeled which doesn't contain the aromatic ring, shows how this procedure could be promising in quantification of larger and more complicated peptide sequences. The same procedure mentioned earlier can be done for quantification of T6/*T6. The peak of Phe also appears in T6 spectrum but since T6 is a longer chain of amino acids, it is observed that other amino acids could show even more prominent peaks than phenylalanine. It is observed that no matter the amino acid be labeled as a single molecule or as a one connected to another 5 amino acids in T13 and another 22 in T6, no changes in the position of the peaks happen.

The validation results are presented as predicted mass fractions of natural Phe and T13, plotted against the corresponding reference values (figure 5 a,b). The outcome of the internal validation is summarized in terms of the root mean square error of cross validation (RMSECV). The average deviation between the predicted and the true compositions yields a measure for the predictive performance of the model, which is converted into the measurement uncertainty $U(c)$.

The RMSECV values correspond to prediction uncertainties of between 1 and 2 % in the case that the model is applied to approximately equimolar analyte-to-spike ratios.

The uncertainty for both 2 series is of the same order as what attained in quantification of a single amino acid. The same prediction process was done for T6 and RMSE of 0.012024 was obtained, the graph not shown here.

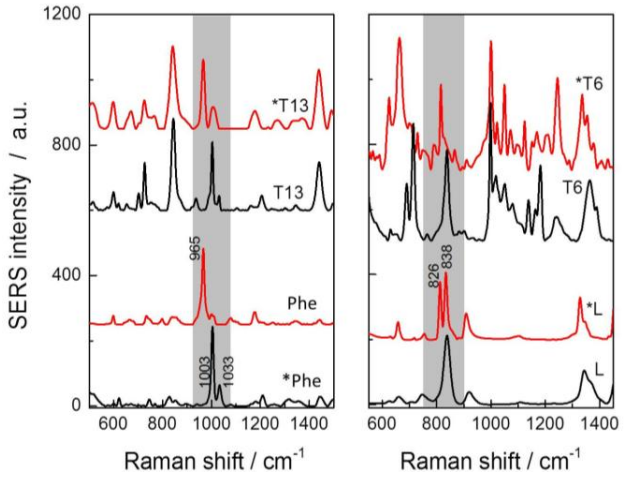

Fig. 4 (a) SERS spectra of natural (Phe) and $^{13}C_9$-^{15}N-labeled phenylalanine (*Phe), natural and *Phe-labeled T13, (b) T6/*T6, T6 based on labeling L

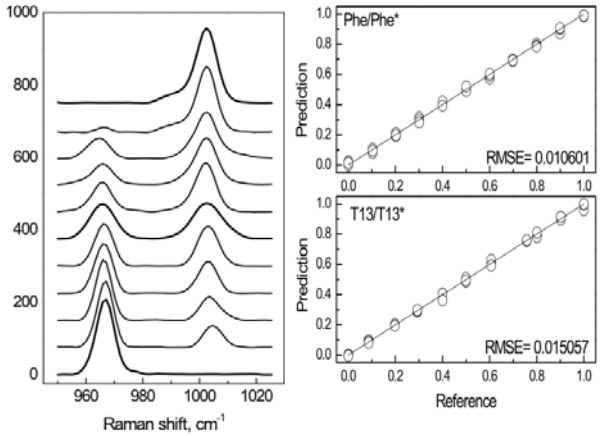

Fig. 5 (a) Full series of calibration spectra recorded to generate the PLS model showing the continuous transition from T13 to *T13; (b) Results of internal cross-validation for two matrixes

It can be observed that extending isotope labeling would be an advantage in order to achieve a lower uncertainty in the quantitative results in comparison with previous results achieved on smaller biomarkers [25].

IV. Conclusion

The present work shows that IDSERS is a sensitive and accurate tool for identification and quantification of signature peptides from complex protein biomarkers. The possibility of quantifying larger biomolecules was also investigated as on-chip assay. As examples, two peptide sequences of the human growth hormone containing 6 (T13) and 22 (T6) amino acids have been analyzed on the basis of the most prominent phenylalanine and leucine bands. The labeled single amino acids, *Phe and *Leu have been investigated here for their use as internal standards for making a reference model of T6 and T13. Having knowledge about molecular binding behavior of a single amino acid and a short peptide on a metal surface leads toward knowing the approximate behavior of longer peptides or even proteins, human growth hormone (hGH) as the example here.

Acknowledgment

Financial supports by the DFG via GRK1570 and IGSM are acknowledged. The Authors gratefully thank Dr. Andre Henrion and Mr. Rüdiger Ohlendorf in PTB and Dr. Gavin O'Connor in LGC, UK, for providing the purified peptide isotopologues.

References

[1] L.Seballos, N. Richards, D. J. Stevens, M. Patel, L. Kapitzky, S. Lokey, G. Millhauser, J. Z. Zhang, Chemical Physics Letters, 2007, 447, 335-339
[2] D.R.J. Bainbridge, S.A. Ellis, I.L. Sargent, J. Reprod. Immunol. 2000, 48, 17.
[3] N. Andreasen, L. Minthon, P. Davidsson, E. Vanmechelen, H. Vanderstichele, B. Winblad, K. Blennow, Arch. Neurol. 58, 2001, 373.
[4] M.A. Albert, E. Danielson, N. Rifai, P.M. Ridker, JAMA 286, 2001, 64.
[5] P. Argani et al., Clin. Cancer Res. 7, 2001, 3862.
[6] K. Ryu, A. J. Haes, H. Park, S. Nah, J. Kim, H. Chung, M. Yoon, S. Han, J. Raman Spectroscopy, 2010, 41, 2, 121–124
[7] S. M. Hewitt, J. Dear, R. A. Star, J. Am. Soc. Nephrol. 2004, 15, 1677.
[8] S. Bi, H. Zhou, S. Zhang, Biosens. Bioelectron. 2009, 24, 2961.
[9] K. A. Jellinger, B. Janetzky, J. Attems, E. Kienzl, J.Cell.Mol.Med. 2008, 12(4), 1094.
[10] F. Wei, D. Zhang, N. J. Halas, and J. D. Hartgerink, J. Phys. Chem. B, 2008, 112, 9158-9164
[11] T. Vo-Dinh, D.L. Stokes, G.D. Griffin, M. Volkan, U.J. Kim, M.I. Simon, J. Raman Spectrosc. 1999, 30, 785.
[12] V. Brun, C. Masselon, J. Garin, A. Dupuis, J. Proteomics, 2009, 72, 740-749.
[13] C. G. Arsene, R. Ohlendorf, W. Burkitt, C. Pritchard, A. Henrion, G. O'Conner, D. M. Bunk, and B. Güttler, Anaytical Chemistry, 2008, 80, 4154-4160
[14] M. D. Porter, R. J. Lipert, L. M. Siperko, G.Wang, R.Narayanana, Chem. Soc. Rev. 2008, 37, 1001.
[15] W. E. Doering, M. E. Piotti, M. J. Natan, R. G. Freeman, Adv. Mater. 2007, 19, 3100.
[16] R. Stosch, F. Yaghobian, T. Weimann, R. J. C. Brown, M. J. T. Milton and B. Güttler, Nanotechnology, 2011, 22, 105303.
[17] S. Stewart, P. M. Fredericks, Spectrochim. Acta, Part A 1999, 55, 1641.
[18] J. S. Suh, M. Moskovits, J. Am. Chem. Soc. 1986, 108, 4711.
[19] T. M. Herne, A. Ahern, R. L. Garrell, J. Am. Chem. Soc. 1991,113, 846.
[20] E. Podstawka, Y. Ozaki, L.M. Proniewicz, Appl. Spectrosc. 2005,59, 1516.
[21] H. I. Lee, S. W. Suh, M.S. Kim, J. Raman Spectrosc. 1988, 19,491.
[22] C. D. Keating, K.M. Kovaleski, M. J. Natan, J. Phys. Chem. B 1998, 102, 9404.
[23] L. A. Gearheart, H. J. Ploehn, C. J. Murphy, J. Phys. Chem. B 2001, 105, 12609.
[24] R. Stosch, A. Henrion, D. Schiel, B. Güttler, Anaytical Chemistry, 2005, 77, 7386-7392.
[25] F. Yaghobian, T. Weimann, B. Güttler, R. Stosch, Lab on a Chip, 2011, 11, 2955-2960.
[26] F. Yaghobian, IDSERS based Quantification of Biomarkers on a Single Chip; Mench & Buch, Berlin, 2011.
[27] T. Tsushima, Y. Katoh, Y. Miyachi, K. Chihara, A. Teramoto, M. Irie, Y. Hashimoto, The Journal of Clinical Endocrinology & Metabolism, 1999, Vol. 84, No. 1 317-322.
[28] C. Ortiz, D. Zhang, Y. Xie, V.J. Davisson, D. Ben-Amotz, Anal. Biochem. 2004, 332, 245.