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Application Note

Biology
Life Sciences
Raman

RA09

Insights into thrombosis mechanisms using high resolution SERS

Within the field of bio-medical research Raman spectroscopy is fast being adopted as a valuable molecular and micro-analysis tool. The detailed vibrational information contained within a spectrum is ideal for probing structural and conformational changes of biologically active compounds, and providing selective markers for identification of disease states.

Unlike Fourier Transform Infra-Red (FTIR) spectroscopy, it benefits from limited interference by water, and is far better suited for confocal analysis. Thus, for analysis within typical water containing biological media with micron spatial resolution, Raman is the technique of choice. Traditionally, the drawbacks of Raman have been its relatively low sensitivity, and potential interference from fluorescence. For biological studies in particular, these have been major stumbling blocks.

However, more recent development of Surface Enhanced Raman Spectroscopy (SERS) overcomes these issues, by offering greatly enhanced sensitivity for samples in close contact with a roughened metal surface. In this application note, SERS is used to study the behaviour of glycoprotein αIIbβ3 (Figure 1) which plays a critical role in thrombosis (pathological blood clotting which can reduce or completely stop blood supply to tissues and organs, leading to myocardial infarction or stroke).

In particular S-S (~500cm^{-1}), C-S (~690cm^{-1}), and S-H (~2570cm^{-1}) marker bands are used to probe chemical changes caused by activation of the protein. These bonds have a strong effect on its 3D structure, and thus are important keys to conformational changes occuring during activation.

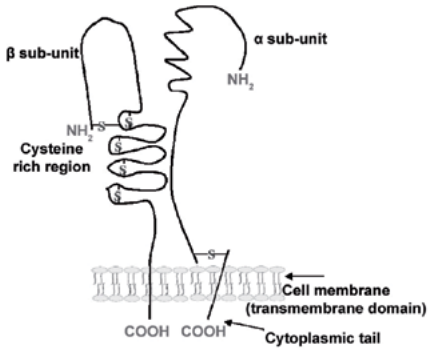

Figure 1 – Representation of the structure of transmembrane protein $\alpha_{IIb}\beta_3$

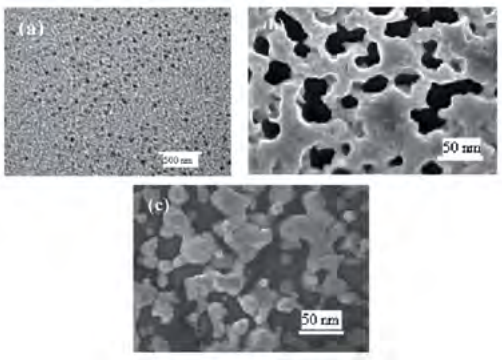

Figure 2 – SEM of (a) nanoparticles deposited on thiolated glass, (b) electrochemically roughened gold surface, and (c) electrodeposited gold islands on gold.

Optimisation of SERS substrates

The SERS measurements discussed herein were made using gold substrates. However, the SERS performance (enhancement factor and reproducibility) of the gold depends on its surface morphology and structure. Thus, initial work concentrated on characterising the SERS effect of gold substrates prepared in different ways. Three methods were chosen: (1) gold nanoparticles on thiolated glass, (2) electrochemically roughened gold, and (3) electrochemically deposited gold on gold. SEM images illustrate the morphogical differences resulting from these three methods (Figure 2).

The substrates were excited using the 632.8nm line from a HeNe laser, and comparison of signal obtained from a 25 µmol solution of L-cysteine was used to judge the most suitable substrate. The analyses were made on a LabRAM HR instrument, and resulted in the electrochemically roughened gold substrate being chosen, on account of its excellent reproducibility, and good enhancement (five orders of magnitude).

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Figure 3 illustrates spectra obtained from solid amino acid (normal Raman), and the 25 µmol solution (SERS). Whilst the two spectra compare well, there are clear differences. For example, in the 500-900 cm^{-1} region of the SERS spectrum the C-S mode is enhanced and red shifted from 690 cm^{-1} to 680cm^{-1} due to strong binding to the substrate. Similarly, the intense S-H vibration observed in the 2500-2600 cm^{-1} region for the solid is lost in the SERS, due to immobilisation of this functional group at the gold surface by formation of S-Au bonds.

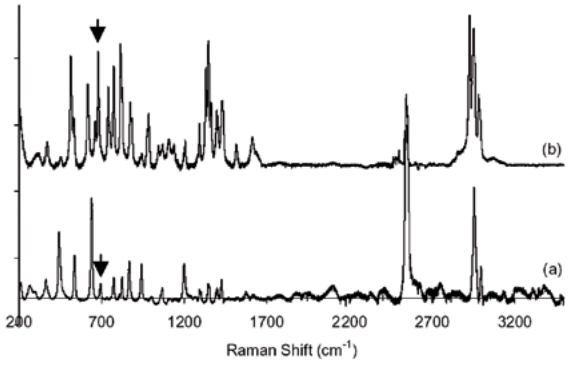

Figure 3 – spectra of (a) solid L-cysteine acquired by normal Raman, and (b) 25 umol L-cysteine acquired by SERS. The C-S bands discussed in the text are indicated with arrows.

Protein analysis

With the optimisation of the SERS substrate complete, the $\alpha_{IIb}\beta_3$ protein in buffered solution was analysed, to characterise conformational changes caused by protein activation. The protein was activated prior to use by incubating with the agonist of choice for 30 minutes (at 30oC). The three most common agonists for this protein were used, namely, dithioreithol (DTT), EDTA and Mn(II).

The SERS spectrum of the native glycoprotein $\alpha_{IIb}\beta_3$ is shown in Figure 4. Its particularly complex nature is due to contribution from both its carbohydrate portion and the protein itself. There are 18 cysteine residues in the α chain, and 56 in the β chain, but these are almost exclusively in the disulfide (S-S) form in the inactive protein.
Thus, there are two main regions of interest in the spectrum - the 300-700 cm^{-1} region containing the disulfide bands, and 1800-2800 cm^{-1} where S-H moieties from this cysteine rich compound would be apparent.

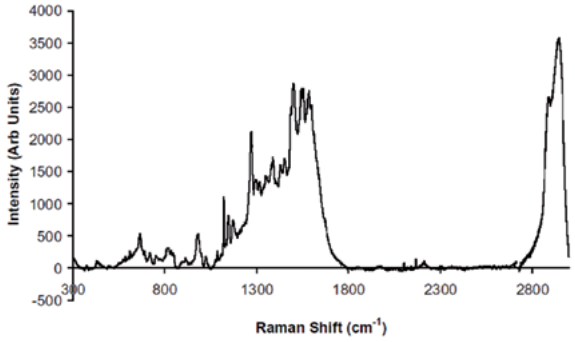

Figure 4 – Raman spectrum of 25 µmol$\alpha_{IIb}\beta_3$ in Tris buffer, deposited on electrochemically roughened gold. Spectrum is background and buffer/solvent corrected.

It is generally assumed that the three agonists (DTT, EDTA and Mn(II)) cause the same response from the protein, albeit by different mechanisms. DTT and Mn(II) are reducing agents, and are believed to reduce a number of disulfide bonds in the cysteine rich region of the protein, thus causing a conformational change. EDTA is thought to chelate Ca^{2+} from the Ca binding sites, eliciting an enzymatic response which leads to disulfide reduction.

Figure 5 shows the Raman spectrum of the native and activated forms of the protein in the disulfide region. With DTT activation, there is a simplification of the complex band centered at 490 cm^{-1}, due to reduced low energy shoulders at 478 cm^{-1} and 488 cm^{-1}. These are attributed to the most "strained" disulfide bonds, and indicates that it is these strained bonds which are preferentially reduced by DTT.

In addition, the C-S mode at 676 cm^{-1} shifts to 681 cm^{-1} on activation, which is attributed to S-H formation. Whereas in L-cysteine no S-H bonding is present due to S-Au bonding to the substrate (see Figure 3), the complex 3D structure of the protein shields the S-H moities

from the substrate and thus it is possible to observe their contribution within the SERS spectrum. Analysis of the S-H region corroborates this (figure 6), with peaks visible at 2571 cm^{-1} and 2590 cm^{-1} in the DTT activated protein spectrum.

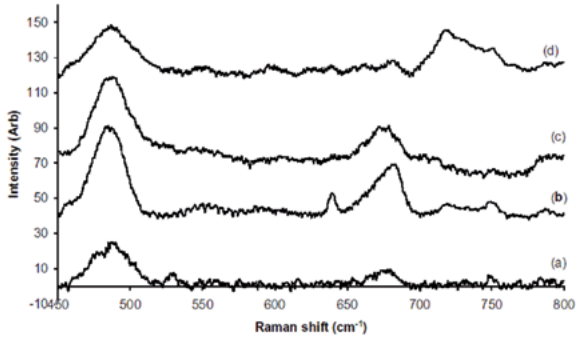

Figure 5 – Raman spectral region 450-800 cm^{-1} (a) native $\alpha_{IIb}\beta_3$, (b) DTT activated $\alpha_{IIb}\beta_3$, (c) Mn(II) activated $\alpha_{IIb}\beta_3$ and (d) EDTA activated $\alpha_{IIb}\beta_3$

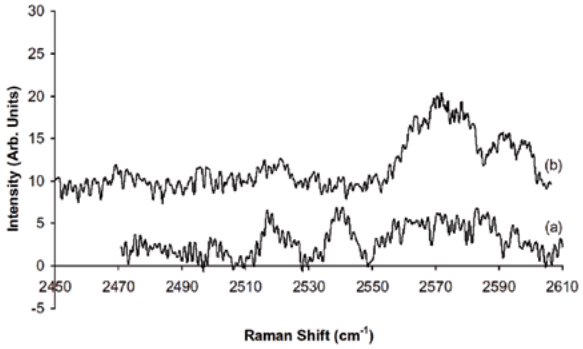

Figure 6 – Raman spectral region 2,450 to 2,610 cm^{-1} (a) native $\alpha_{IIb}\beta_3$, (b) DTT activated $\alpha_{IIb}\beta_3$,

With Mn(II) activation, a similar sharpening of the intense 490 cm^{-1} feature caused by reduction of strained disulfide bonds is observed, as with DTT. However, the blue shift of the 676 cm^{-1} band isn't apparent, and suggests that Mn(II) causes partial activation. This is an important conclusion, since Mn(II) has been widely regarded as inducing complete activation.

Finally, in the EDTA activated protein there is no observable change in the 490 cm^{-1} band, although the intensity of the 680 cm^{-1} C-S mode is considerably weakened. A new feature is apparent at 719 cm^{-1}, the origin of which is currently unclear - however, it may be associated with a strained C-S linkage. It is suggested that there are changes to the disulfide bonds, but not to the highly strained ones.

This would look to agree with previous work that EDTA activation occurs through a different mechanism from the reduction mechanism of DTT and Mn(II). It is possible that activation of EDTA does occur through the enzymatic process mentioned above – if so, it would appear to be through reduction of only a few cysteine residues. Since the Raman spectrum corresponds to 74 residues, the reduction of just one or two residues may not be apparent. In contrast, DTT and Mn(II) reduction processes are relatively gross, and thus are clearly observed. Quantitative analysis of these results has not been attempted, since measurements were made on bare metal substrates - this gives a chance of newly formed S-H groups being exposed to the metal surface due to structural changes in the protein. Subsequent work using protected SERS platforms is ongoing, allowing more detailed study of these mechanisms.

Summary

$\alpha_{IIb}\beta_3$ is a key mediator in the thrombotic process and has been studied extensively. This current work reports the first Raman spectrum of the protein, and examines changes in conformation during activation. Using three common agonists (DTT, Mn(II) and EDTA), the results indicate that in each case activation is caused by reduction of disulfide links, but suggests that different disulfides are activated according to the reagent used. The histopathological correlation to these findings has yet to be determined.

Acknowledgements

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Reference

Tia E. Keyes, Deirdre Leane, Robert J. Forster, Niamh Moran, Dermot Kenny, New Insights into the molecular mechanisms of thrombosis from high resolution surface enhanced Raman microscopy, Proceedings of the International Society for Optical Engineering, 2005, 5826, 221

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