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Confocal Raman and fluorescence spectroscopy applied to polymeric chromatographic adsorbent particles

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Abstract

Distributions of functional groups in individual chromatographic adsorbent particles have been investigated using confocal Raman and fluorescence spectroscopy. Measurements have been performed with different microscope objectives and it was found that an immersion objective should be used when wet adsorbent particles are analysed. Nd^{3+} ions have been used as fluorescence probes to evaluate the distribution of chelating and negatively charged functional groups. The fluorescence spectrum from Nd^{3+} has also been used to obtain information about the coordination of Nd^{3+} within the adsorbent particles.

Keywords: Confocal Raman spectroscopy; Adsorbents; Chelating Sepharose Fast Flow; Agarose

1. Introduction

Confocal Raman spectroscopy combines the chemical information from vibrational spectroscopy with the spatial resolution of confocal microscopy. Depth profiles or lateral Raman mappings can be recorded by moving the sample through the focus of the microscope objective. In this way it is possible to monitor the distribution of different components of a specimen without complicated sample preparations. Confocal Raman spectroscopy has already efficiently been used for depth profiling and lateral mapping of membranes, laminates and coatings [1–6]. Coatings and thin films which have been subjected to surface modifications have been characterised by spatially mapping the distribution of additives or different ingredients of a formulation [1,2]. Structural changes of a fuel cell membrane, prior and after fuel cell testing, have been studied to obtain information about the degradation of the membrane [3]. Investigations of the distribution of small molecules in latex or poly(vinyl chloride) (PVC)-films have been performed [4,5]. Orientation profiles of amorphous and crystalline phases across a polyethylene fibre section have also been evaluated with confocal Raman spectroscopy [6].

Recently we have used confocal Raman spectroscopy to examine distributions of functional groups in individual chromatographic adsorbent particles [7]. The distribution of immunoglobulin G (IgG) antibodies immobilised on CNBr activated agarose particles was investigated and it was found that the IgG antibodies had an even distribution within the adsorbent particles. Knowledge about the distribution of functional groups in chromatographic

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adsorbent particles is of great interest. Too high a concentration of functional groups at the outer layer of the particles might lead to crowding, i.e., the functional groups are situated so close to each other that simultaneous binding to all functional groups is impossible for sterical reasons. If such a crowding occurs the capacity of the gel will be reduced. A high density of ligands may also have an influence on the kinetics, promote avidity effects in the binding of large molecules, or lead to association of smaller ones [8,9]. Examples with an uneven distribution have been reported [10,11]. Subramaniam et al. [10] studied the role of antibody density effects on immunosorbent efficiency. Fluorescent-labelled beads were sectioned using a microtome, and labelled antibodies were then visualised by immunofluorescence. McAlpine and Screiber [11] used optical analysis for indirect measurement of the distribution of a fluorescent dye covalently attached to the particles. Detection at the wavelength-specific emission of the fluorophore allowed visualisation of the dye distribution. Another technique that has been applied is confocal scanning laser microscopy (CSLM). The distribution of immobilised antibodies was measured indirectly via saturation with fluorescent labelled protein A [7]. CSLM has also been used for studies of trypsin immobilisation on porous glycidyl methacrylate beads [12]. Confocal Raman spectroscopy allows a direct measurement of the distribution of functional groups since it is possible to follow the Raman signals of the functional group. However, when confocal settings are used only a small amount of the Raman scattered signal is detected and the signals obtained are lowered. Hence, longer measuring times then for conventional Raman spectroscopy are needed. In the present work we have found that Nd^{3+} ions can be used as fluorescence probes and the intensity variation of the fluorescence signal can be used in the evaluation of depth or lateral profiles. Limiting factors are of course that the use of Nd^{3+} demands the presence of negatively charged functional groups or chelating groups that are able to attach the neodymium ions and that an indirect measure of the profile is obtained, in the same sense as with CSLM. An advantage is however that Nd^{3+} can be used in those cases where the spectrum of the functional group does not significantly differ from the spectrum of the matrix. The fluorescence spectrum can also be used to obtain information of changes in the coordination of the neodymium ions. The fine structure of the bands in the absorption spectra and fluorescence spectra have been shown to be dependent on the local electric field around the ions [13–18]. When the coordination of the ions were changed, shifts could be observed in these spectra. The small size of the Nd^{3+} ensures that all functional groups can be reached. The fluorescence signal from the hydrated Nd^{3+} ion is pH independent over a large pH interval making any buffering, needed for proteins, unnecessary when only a distribution of functional groups is investigated.

In the literature a depth resolution in confocal Raman spectroscopy of typically 2–5 μm have been quoted [1–3]. The depth resolution is often measured by recording the intensity of the Si peak at 520 cm^{-1} as a function of the distance to a silicon wafer. The full-width-at-half-maximum (FWHM) of the recorded profile is taken as a measure of the depth resolution. It has, however, been emphasised in recent articles by Everall [19,20] that, if metallurgical objectives are used to focus below the surface of the sample, refraction at the air–sample surface may occur. The effect of the refraction is to distort the laser focus and to shift the focus deeper into the sample. Everall suggests that unless the effects of refraction are accounted for quantitatively, inaccurate conclusions regarding interface and layer position/thickness can be inferred from raw *z*-scan intensity data. To avoid the problem with refraction at the sample/air surface an immersion objective with a suitable liquid that matches the refractive index of the sample has to be used. If no objective is available profiles can be obtained by cross sectioning of the sample followed by lateral scanning [19,20].

In this paper we have evaluated how different objectives affect the resulting intensity profiles (depth or lateral). We have further shown how Nd^{3+} can be used to probe the distribution of functional groups in chromatographic adsorbent particles.

2. Experimental

The spectroscopic data were obtained with a Renishaw 2000 Raman spectrometer equipped with a

near-infrared diode laser (783 nm, 20 mW). In the experiments two different objectives were used, a dry metallurgical 50×, 0.75 NA objective and a water immersion 63×, 0.90 NA objective.

The adsorbent particles were placed on a plate of stainless steel during the measurements. When the adsorbent particles were measured wet they were covered by a cover glass. A simplified sketch of the experimental set up for measurements of wet adsorbent particles is shown in Fig. 1. An *x*-, *y*-, *z*-motorised stage was used for the stepwise scanning in the confocal measurements. The Nd^{3+} ions were immobilised to the adsorbent particles by immersing the particles in a 0.05 *M* $Nd_2(SO_4)_3$-solution for 24 h. To ensure that possible excess of Nd^{3+} was removed the particles were rinsed in distilled water afterwards and soaked in distilled water for at least 24 h before the measurements.

2.1. Samples

Two different types of adsorbent particles have been investigated. Both samples were obtained from Amersham Biosciences. The first sample, Chelating Sepharose Fast Flow, intended for immobilised metal ion affinity chromatography [21–23], is based on cross-linked agarose with functional groups consisting of chelating iminodiacetic groups. The distribution of the chelating groups was expected to be even in these particles. The second sample was agarose particles with an uneven distribution of negatively charged sulfopropyl groups (not commercially available) [24,25]. The sulfopropyl groups were expected to be located to a surface layer.

The highly porous character of wet agarose particles makes them optically very similar to water. Thus, no signal decrease due to absorption could be observed when the measurements were performed in wet conditions.

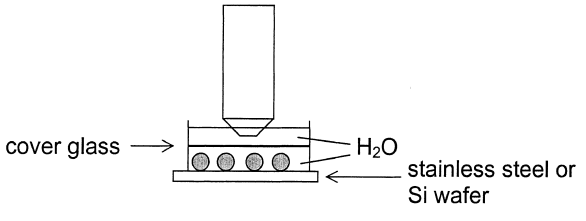

Fig. 1. Schematic figure for the experimental set up for measurement of wet adsorbent particles.

3. Results and discussion

3.1. Intensity profiles

3.1.1. Depth profiles of Si

Depth profiles where the intensity of the 520 cm^{-1} band of silicon was followed were recorded, both with the dry metallurgical objective and with the immersion objective. To compare the performance of the objectives in wet measurements the Si signal was followed through a wet adsorbent particle, which was placed, on a silicon wafer. The resulting depth profiles are shown in Fig. 2a and b. For comparison the depth profile of silicon in air measured with the dry metallurgical objective is included in Fig. 2c. The depth resolution was determined from the computation of the FWHM of the intensity profile. The FWHM of the intensity profile measured in air, with the dry metallurgical objective, was ~6 μm and in water, measured with the immersion objective, approximately 8 μm. Hence, a slightly poorer depth resolution was obtained for the measurement in water. However, when comparing the depth profiles measured through water and a particle, using different objectives, it can be seen that the profile from the immersion objective is more narrow and symmetrical whereas the profile from the dry metallurgical objective is wider and unsymmetrical. This observation is in accordance with Everall [19,20], which states that if a dry metallurgical objective is used to focus below the surface of a transparent material (in our measurement an adsorbent particle) the point of focus will be shifted deeper into the sample and the depth resolution will decrease. Thus, to get reliable results when measuring wet particles a water immersion objective should be used.

3.1.2. Intensity profiles using Nd^{3+}

The fluorescence signal from Nd^{3+} consists of several overlapping bands as can be seen in Fig. 3 for Nd^{3+} in water and Nd^{3+} attached to chelating groups in Sepharose particles. The signal is attributed to the transition $^4F_{3/2} \rightarrow {}^4I_{9/2}$ [14,18] and the fine structure is due to the Stark splitting by the local

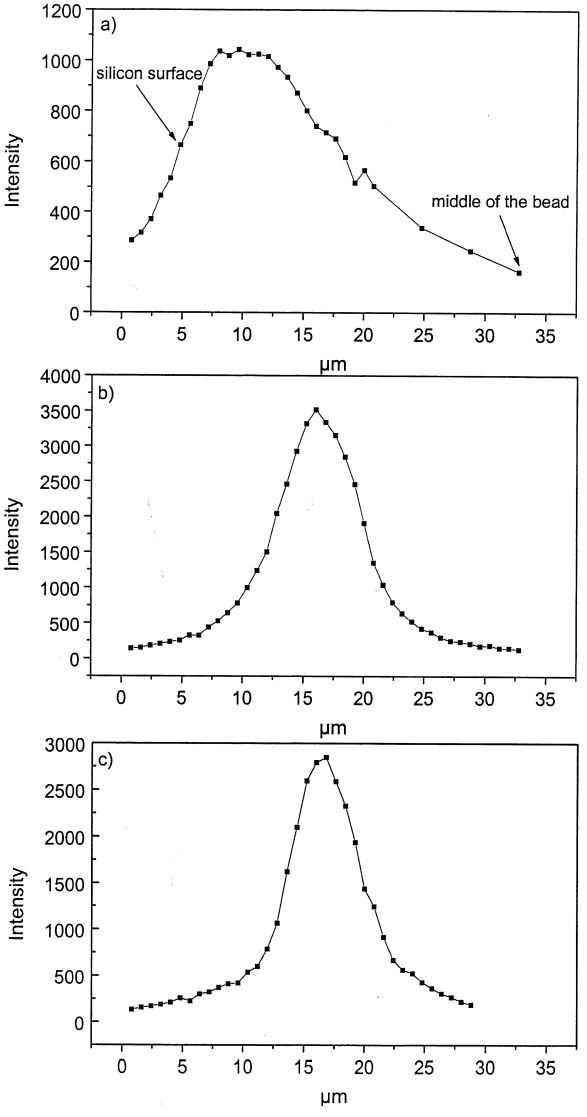

Fig. 2. Intensity of Raman Si-peak at 520 cm^{-1}. (a) Measured with a dry metallurgical 50×-objective through water and an adsorbent particle. (b) Measured with a water immersion 63×-objective in water through an adsorbent particle. (c) Measured with a dry metallurgical 50×-objective in air. The zero-point at the *x*-axis indicates where the measurement was started.

field. Since for a given sample the fluorescence signal was found to have the same appearance, the intensity of the strongest band was used in the profile plots.

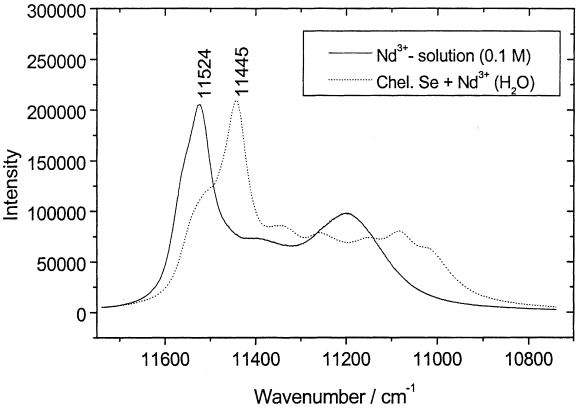

Fig. 3. Fluorescence spectra from Nd^{3+} in water solution and in Chelating Sepharose Fast Flow.

3.1.2.1. Chelating Sepharose Fast Flow

In the Chelating Sepharose Fast Flow Nd^{3+} was attached to the chelating iminodiacetic groups. Depth profiles showing the distribution of Nd^{3+} in Chelating Sepharose Fast Flow is shown in Fig. 4a and b. Measurements have been performed both from side to side through the centre and from bottom to top of the spherical particles. The depth profiles in Fig. 4a and b are measured with different objectives. The depth profiles obtained with the dry metallurgical objective (Fig. 4a) show a maximum of the Nd^{3+} signal in the middle of the particle whereas a flat depth profile of Nd^{3+} is obtained with the immersion objective (Fig. 4b). This flat profile was the expected result, which was also obtained when the particles were measured dry. The increase of the Nd^{3+}-signal in the middle of the particle when using the dry metallurgical objective could be explained by the increase of the focal volume when focusing into a material of a different refractive index [19,20]. The effect can be seen in the measurement from side to side. In the edge of the particle a small amount of the particle and a larger amount of the surrounding water is included in the focal volume and the measurement gives a low Nd^{3+}-signal. In the middle of the particle the focal volume is exclusively from the particle and hence a large Nd^{3+}-signal can be obtained. When the immersion objective is used the focal volume is smaller and no differences of the

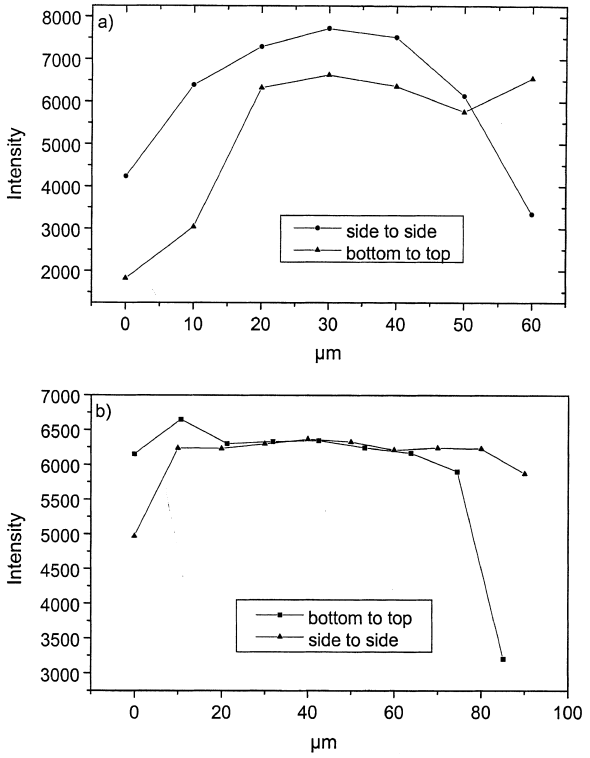

Fig. 4. Intensity profile of Nd^{3+} fluorescence. (a) In a Chelating Sepharose Fast Flow particle measured with a dry metallurgical 50×-objective. (b) In a Chelating Sepharose Fast Flow particle measured with a water immersion 63×-objective.

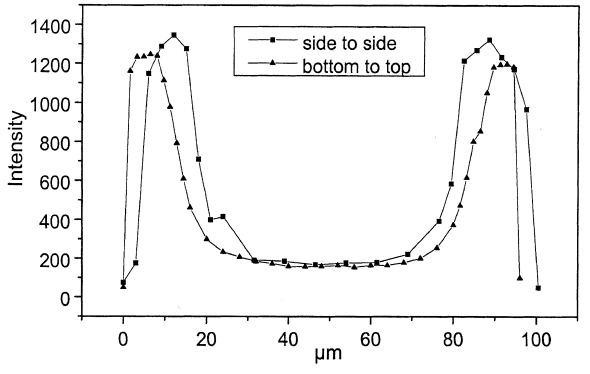

Fig. 5. Intensity profile of Nd^{3+} fluorescence in an agarose particle with a surface layer of sulfopropyl groups measured with a water immersion objective.

middle and the edge can be found. These results again show the importance of using an immersion objective when wet adsorbent particles are examined.

3.1.2.2. Agarose particles with a surface layer of sulfopropyl groups

Agarose particles with a surface layer of sulfopropyl groups were used to investigate the applicability of the confocal spectroscopic method. Nd^{3+} was attached to the negatively charged sulfopropyl groups, which made it possible to follow the distribution of sulfopropyl groups by the distribution of Nd^{3+}. Measurements were performed both from side to side and from bottom to top. In Fig. 5 the resulting profiles are given. As can been seen the distribution show the expected appearance with a decrease of sulfopropyl groups in the middle of the particle. The thickness of the surface layer containing sulfopropyl groups can be estimated to 15–20 μm. Control measurements were performed on agarose particles without sulfopropyl groups. When the measuring times were increased three times, compared to measurements on agarose particles with sulfopropyl groups, an extremely low fluorescence signal from Nd^{3+} was obtained. This low signal was estimated to be approximately 3% of the strongest signal from the agarose particle with sulfopropyl groups. No variation in intensity was found when measurements were performed from side to side of the particle. The presence of charged groups may be due to small amounts of naturally-occurring acidic groups in the raw materials (e.g., sulfate groups in agarose or carboxylic acid groups in dextran) [26].

3.1.3. Intensity profiles using the Raman signal from sulfopropyl groups

Lateral profiles of the agarose particles with a surface layer of sulfopropyl groups were also analysed with confocal Raman spectroscopy. In Fig. 6 reference spectra of agarose and agarose coupled with sulfopropyl groups are shown. As can be seen the Raman signal from the sulfopropyl groups differs from the Raman signal of agarose. The intensity variation of the Raman signal from the sulfopropyl groups (symmetric SO_3^- stretching band) was followed in measurements performed from side to side of the particle. The resulting lateral profile is shown

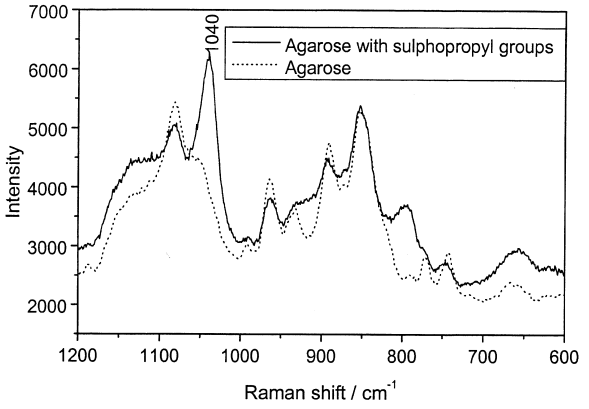

Fig. 6. Raman spectra of agarose and agarose coupled with sulfopropyl groups.

in Fig. 7. The profile shows the expected result with high intensities from the sulfopropyl groups at the surface and lower intensities in the middle of the particle. The measurement was performed in water using the immersion objective. The measurement demanded long measuring times, approximately 1 h for each scan point. By using confocal Raman spectroscopy a direct measure of the distribution of sulfopropyl groups is obtained, on the other hand the measuring times are prolonged from 30 s for each scan point in the Nd^{3+} experiment to 1 h per scan point in the Raman experiment.

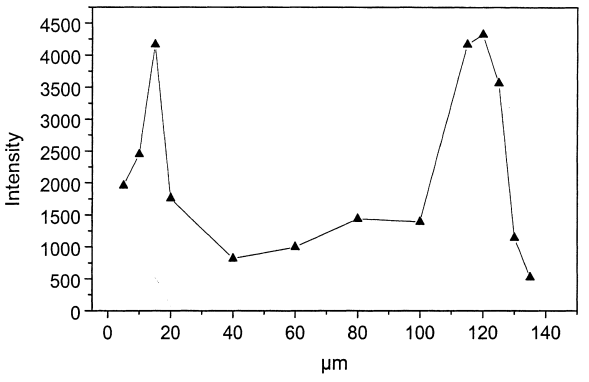

Fig. 7. Intensity profile of the Raman signal from sulfopropyl groups in an agarose particle measured with a water immersion objective from side to side of the particle.

3.2. Nd^{3+} as an environmental probe

Nd^{3+} cannot only be used as an indication of the presence of negatively charged groups but also to obtain information about the surroundings of the Nd^{3+} ions. Fluorescence spectra of Nd^{3+} were recorded with Nd^{3+} in different environments. In Fig. 3 spectra obtained with Nd^{3+} in water and of Chelating Sepharose Fast Flow saturated with Nd^{3+} in water are shown. When comparing these fluorescence spectra changes can be observed, which are caused by different coordination of the neodymium ions. Smaller changes can also be found when comparing the fluorescence spectra from Nd^{3+} with Chelating Sepharose Fast Flow in a buffer solution (20 m*M* phosphate and 150 m*M* NaCl, pH 7.4) with or without addition of 500 m*M* imidazole (Fig. 8). In order to strengthen the changes in the fluorescence spectra evaluation with second derivative analysis was performed. This procedure makes it easier to resolve the changes in the spectra from Nd^{3+} in water and in buffer (Fig. 9a). These changes indicate that Nd^{3+} might coordinate to some of the negatively charged ions in the buffer solution, i.e., Cl^-, $H_2PO_4^-$ or HPO_4^{2-}. As can be seen in Fig. 9b it is also possible to detect changes in the spectra from Nd^{3+} in Chelating Sepharose Fast Flow in buffer with or without imidazole. The shift in the fluorescence spectra from Nd^{3+} that occurs upon adsorption of

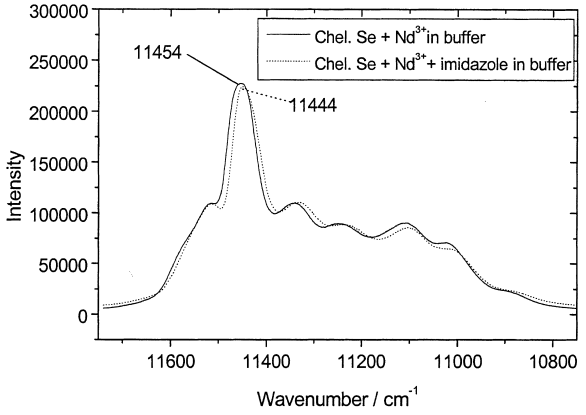

Fig. 8. Fluorescence spectra from Nd^{3+} with Chelating Sepharose Fast Flow in buffer and buffer+imidazole.

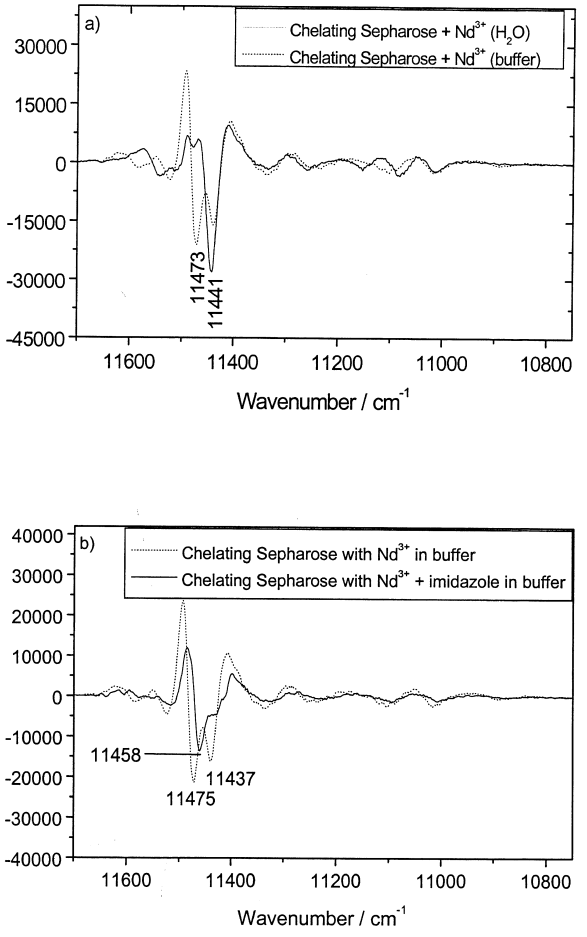

Fig. 9. Second derivative analysis of the fluorescence spectra from Nd^{3+}. (a) With Chelating Sepharose Fast Flow in water and in buffer. (b) With Chelating Sepharose Fast Flow in buffer and buffer+imidazole.

imidazole gives an opportunity to detect if imidazole is coordinated to Nd^{3+}.

In protein purification by immobilised metal affinity chromatography the incoming protein binds to the metal ions via electron donor groupings resident on its molecular surface [22]. Peptides containing histidine residues form stable coordination compounds with metal ions due to participation of imidazole side-chains in chelation. In our experiment we have used imidazole to mimic the adsorption of protein to the immobilised metal ions. Since shifts in the Nd^{3+} spectra can be observed when imidazole is adsorbed it should also be possible to analyse how proteins are distributed in the adsorbent particle by performing a depth profile of Nd^{3+}-fluorescence spectra. Another issue of interest is whether the adsorbed proteins occupy all available sites or if there exists both occupied and unoccupied sites. This can be done by careful analysis of the fluorescence spectra where the contributions from the 11 454 cm^{-1} and the 11 444 cm^{-1} bands (Fig. 8) are calculated. This simple example can in principle be generalised to other types of chelating groups, like *O*-phospho-L-serine and 8-hydroxyquinoline, where other coordinate sites on a protein can be involved [27,28].

4. Conclusions

In the present work it has been shown that an immersion objective should be used if measurements are performed on wet particles. Otherwise, refraction effects may arise and false distributions of functional groups may be obtained. It has been found that Nd^{3+} ions can be used as fluorescence probes and the intensity variation of the fluorescence signal can be used in the evaluation of depth or lateral profiles of functional groups. It is also possible to get a direct measure of the distribution of functional groups by using confocal Raman spectroscopy, however the measurement times are much longer. Investigations of the fluorescence spectra of Nd^{3+} have also shown that it is possible to detect changes in the local environment of Nd^{3+} by observing changes in the fluorescence spectra.

Acknowledgements

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