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Confocal microscopy as a tool for studying protein adsorption to chromatographic matrices

Anders Ljunglöf*, Rolf Hjorth

Pharmacia Biotech AB, S-751 82 Uppsala, Sweden

Abstract

Confocal scanning laser microscopy was used for studying protein adsorption to affinity chromatography matrices. The adsorption of Protein A to IgG Sepharose 6 Fast Flow was studied by batch incubation with varying amounts of fluorescently labeled Protein A. At low sample amounts, Protein A had been adsorbed to a thin outer layer. By increasing the sample–IgG Sepharose ratio, the adsorption layer also increased. Likewise, the adsorption depth was dependent on the incubation time. Finally, a stack of confocal images separated in space was used for three-dimensional reconstruction of the adsorption pattern in a particle.

Keywords: Adsorption; Confocal scanning laser microscopy; Proteins; Protein A

1. Introduction

The adsorption of proteins onto different types of chromatographic matrices has been studied extensively. Most of these studies have been carried out as batch, or column, experiments in which the adsorbent is considered as a bulk. In such experiments, the equilibrium and kinetic characteristics of adsorption can be studied [1], as can the thermodynamics of the process [2,3]. Until very recently [4,5] no attempts to study the adsorption process on a single particle level had been made. Subramanian et al. [4] studied the role of antibody density effects on immunosorbent efficiency. Fluorescently labeled beads were sectioned using a microtome, and labeled antibodies were then visualized by immunofluorescence. Kim et al. [5] have shown that it is possible to monitor the adsorption of a fluorescent molecule at the level of a single particle, on a polymeric cation exchanger using confocal scanning microscopy.

In a confocal scanning microscope detecting fluorescent light from the specimen, the depth-discriminating property can be used to carry out virtual optical sectioning of a specimen. Optical sectioning is a scientifically accepted description of the way a confocal microscope acquires an image [6,7]. The term is intended as an analogy to mechanical sectioning with a microtome [4], which is an alternative technique for obtaining images with sufficient resolution. Mechanical sectioning, on the other hand, is destructive and much more time-consuming. Confocal microscopes achieve high resolution of a selected plane in a specimen by means of three basic steps.

First, light is focused by an objective lens into an hourglass-shaped beam so that the bright "waist" of the beam strikes one spot at some chosen depth in a specimen (Fig. 1). Second, reflected or fluorescent light from that spot is focused to a point and allowed to pass, in its entirety, through a pinhole aperture in a

*Corresponding author.

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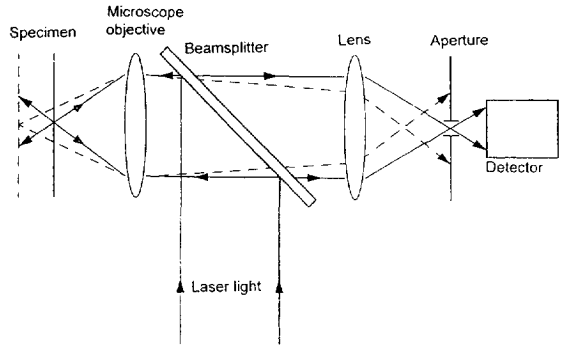

Fig. 1. Simplified ray paths in a confocal microscope. Reflected or fluorescent light from sections of the specimen located out-of-focus will largely fall outside the aperture and is thus effectively blocked. This property makes it possible to slice the specimen optically into thin sections.

mask positioned in front of a detector, which effectively blocks light from out-of-focus planes. Finally, the light is moved from point to point in the specimen until the entire plane of interest has been scanned. The basis of confocal scanning microscopy and its applications have been extensively described [8].

In the present work, direct observation of the adsorption of fluorescently labeled Protein A to an affinity matrix has been explored. *Staphylococcal* Protein A is a receptor protein with primary binding activity restricted to the Fc region of IgG [9,10]. Protein A is structurally and functionally pentavalent in IgG binding [11], and IgG has two functional Fc binding sites available for binding to Protein A [12–14]. These binding properties are utilized in affinity chromatography using immobilized Protein A for purifying antibodies [15,16], and immobilized IgG for the purification of Protein A [11] and Protein A-fusion conjugates produced in prokaryotic expression systems [17,18].

In this work, confocal laser microscopy was used as a tool for studying the adsorption of Protein A to IgG Sepharose 6 Fast Flow. The affinity chromatography gel is based on Sepharose 6 Fast Flow gel matrix, with covalently coupled human IgG. The spherical beads, consisting of cross-linked agarose (6%), has a bead diameter of 45–165 μm in wet form. The medium binds a minimum of 2 mg of Protein A per ml of drained gel, and the exclusion limit for globular proteins on the base matrix, Sepharose 6 Fast Flow, is M_r 4×10^6.

2. Experimental

2.1. Instrumentation

Confocal microscopy analysis was performed with a MultiProbe 2001 Confocal Laser Scanning Microscope (CSLM) supplied with an argon/krypton laser and with ImageSpace Software from Molecular Dynamics (CA, USA).

2.2. Reagents

Sepharose 6 Fast Flow, IgG Sepharose 6 Fast Flow, PD-10 column Sephadex G-25 M and FITC–Protein A were obtained from Pharmacia Biotech (Uppsala, Sweden). FluoroLink Cy5 reactive dye was from Biological Detection Systems (PA, USA).

2.3. Adsorption of Protein A to IgG Sepharose

FITC–Protein A (0.2–5 mg/ml in 20 m*M* sodium phosphate, pH 7.0) was mixed with IgG Sepharose and was gently shaken. The sample–IgG Sepharose ratio was varied between 0.1–5 times of the total Protein A binding capacity, as shown in Table 1. The reaction was allowed to continue for 1 or 10 min (continuous rocking) and was then stopped by dilution and centrifugation, after which the gel was washed three times by repeated dilution, centrifugation and decantation.

2.4. Fluorescent labeling of IgG Sepharose

As a control experiment for the presence of immobilized IgG in the entire Sepharose bead, the beads were labeled with Cy5 as follows.

IgG Sepharose 6 Fast Flow and non-substituted

Table 1
FITC–Protein A–IgG Sepharose incubation

FITC–Protein A (μl, mg/ml)		IgG Sepharose (μl)	Sample–IgG Sepharose ratio[a]
50	0.2	50	0.1
50	2.0	50	1
50	5.0	62.5	2
100	5.0	50	5

[a] Based on a protein A binding capacity of 2 mg/ml of drained gel (IgG Sepharose Fast Flow, Instructions for use).

Sepharose 6 Fast Flow were washed with 0.1 *M* sodium carbonate, pH 9.3, (conjugation buffer) by repeated dilution, centrifugation and decantation. One vial of Cy5, containing sufficient reactive dye to label 1 mg of protein, was diluted in 1 ml of conjugation buffer and divided into two equal parts. A 250-μl volume of IgG Sepharose was added to one part (500 μl) and 250 μl of non-substituted Sepharose were added to the other part. The reaction mixtures were incubated at room temperature, using continuous rocking for 30 min and then the gels were washed three times by repeated dilution, centrifugation and decantation.

These beads were not used for binding experiments with FITC–Protein A.

2.5. Confocal microscopy

A confocal laser scanning microscope, supplied with an argon/krypton laser as shown in Fig. 1, was used for direct observation of fluorescently labeled proteins adsorbed to, or immobilized onto an affinity gel matrix. Gel beads were analysed by section scanning (a single, two-dimensional confocal image) and/or vertical scanning (a single, two-dimensional image perpendicular to a section, parallel to the optical axis). The resulting confocal images were further analysed by use of fluorescence intensity profiles along the particle diameters. A fluorescence intensity profile is the pixel[1] values in a digitised section along a user-defined area, displayed in a diagram. The electrical signal from the detector is digitised to a numerical value between 0–250 arbitrary units (AU).

Furthermore, a three-dimensional volume was described by a stack of confocal images that were separated in space (section series). This was performed by automatic section scanning through a whole bead at levels that were gradually deeper. The step size between the images was set to 10 μm. The laser energy and the sensitivity of the photomultiplier were kept constant during section series.

An objective lens, with a magnification of 60 times, was used in all measurements. The pinhole aperture was set to 50 μm. The fluorophores used in the study was FITC (coupled to Protein A) and the reactive dye, Cy5, which was used for the detection of immobilized IgG. FITC was detected at 488 nm and Cy5 at 647 nm.

Details from each measurement are shown in the text corresponding to the appropriate figure.

3. Results and discussion

Confocal microscopy analysis using section scanning of individual IgG Sepharose beads showed that, at sample amounts corresponding to the Protein A binding capacity of the gel (sample–IgG Sepharose ratio=1), Protein A had been adsorbed to a thin outer layer while the inner part of the bead was unused for adsorption (Fig. 2). To determine if this phenomenon was due to a lack of IgG inside the bead, IgG Sepharose was fluorescently labeled with Cy5 (using Sepharose 6 Fast Flow as a reference) and analysed by section- and vertical scans. The results showed that IgG was distributed throughout the whole bead (Fig. 3a–b), while the background of Cy5 was negligible on non-substituted Sepharose.

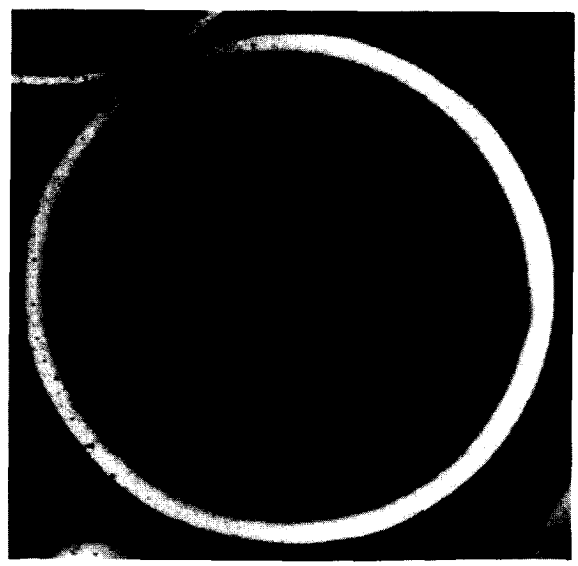

Fig. 2. Confocal image of a 120-μm particle. Sample–IgG Sepharose ratio=1; incubation time, 1 min; laser, 0.5 mW, 488 nm; photomultiplier, 710 V. Section scan: Image size, 512×512 pixels; pixel size, 0.42 μm.

[1] A pixel is a two-dimensional picture element in a confocal image, and the pixel size is related to the distance between scanned points.

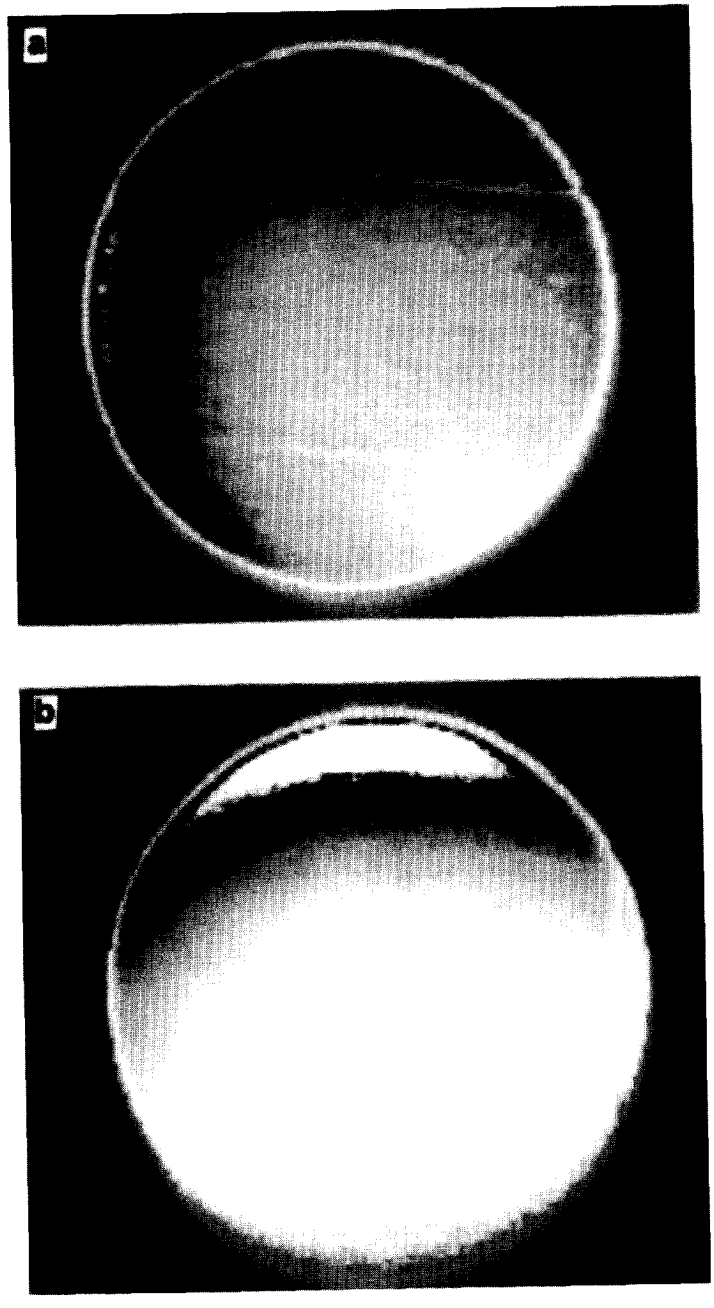

Fig. 3. Confocal image of fluorescently labeled IgG Sepharose (100 μm particle). Laser, 1.6 mW, 488 nm; photomultiplier, 620 V. Image size, 256×256 pixels; pixel size, 0.9 μm. (a) Section scan. (b) Vertical scan.

The concentration of IgG is expected to have a uniform depth around the whole bead. Thus, the weaker intensity obtained at deeper layers is probably due to absorption and scattering of both the excitation and the fluorescent light [19,20].

Fig. 4a–c shows confocal images of IgG Sepha-

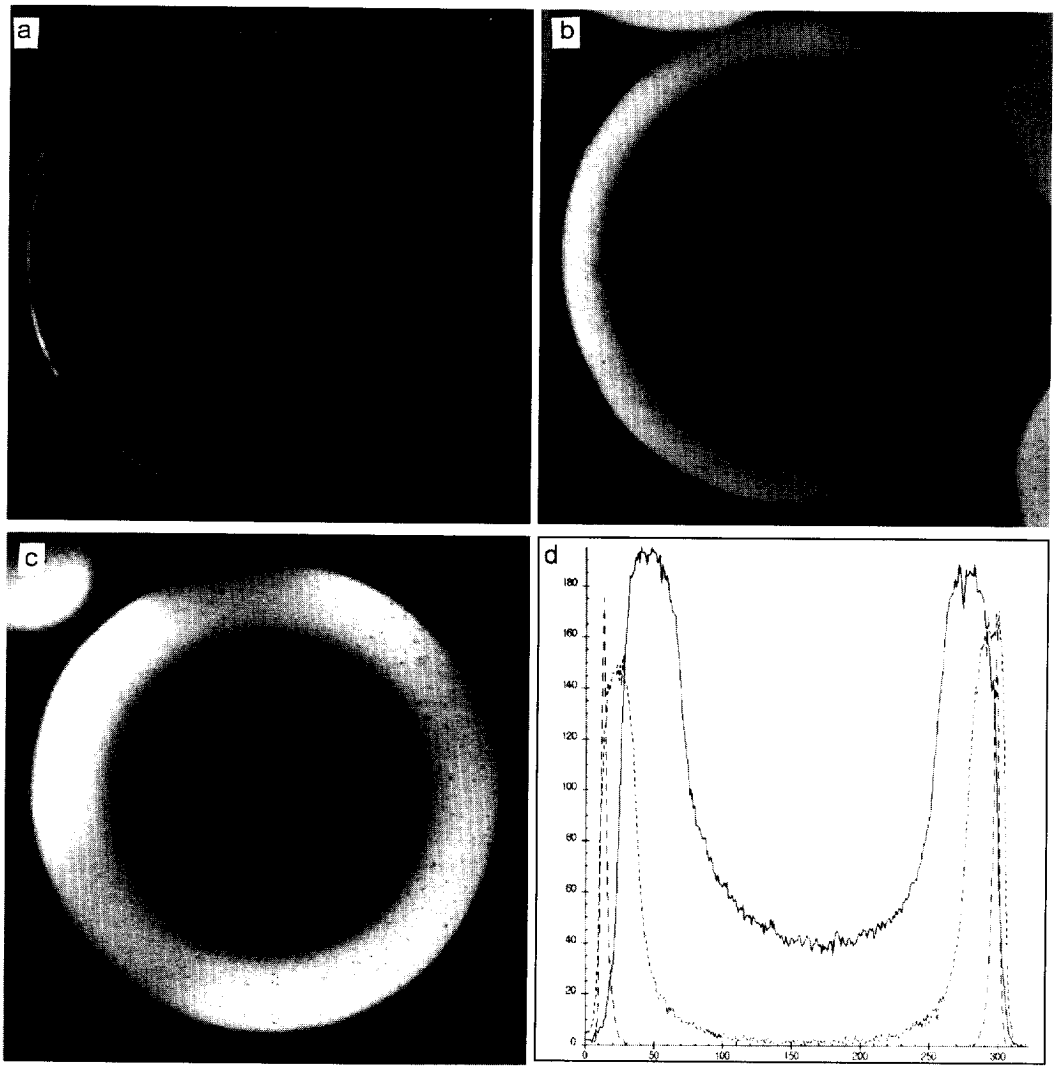

Fig. 4. Confocal image of a 120-μm particle. Incubation time, 1 min; laser, 0.4–1.2 mW, 488 nm; photomultiplier, 635–710 V. Section scan: Image size, 512×512 pixels; pixel size, 0.42 μm. (a) Sample–IgG Sepharose ratio=0.1. (b) Sample–IgG Sepharose ratio=2. (c) Sample–IgG Sepharose ratio=5. (d) Fluorescence intensity profile: x-axis shows pixels and y-axis shows the intensity (in arbitrary units), – – –=a, ···=b, ———=c.

rose 120 μm beads that were incubated for 1 min with FITC–Protein A. At a low sample–IgG Sepharose ratio (0.1), Protein A was adsorbed to a thin outer layer. The fluorescence intensity profile (Fig. 4d) shows that the inner part of the bead was unused for adsorption. By increasing the sample–IgG Sepharose ratio to 2, the adsorption layer also increased, while the inner part of the bead still was unused. At a ratio of 5, the adsorption layer was further increased and also the inner part of the bead was partially utilized for adsorption.

Confocal images of 50 μm beads are shown in Fig. 5a–c. As above, the fluorescence intensity profile (Fig. 5d) reveals that, at a sample–IgG Sepharose ratio of 0.1, Protein A was adsorbed only to a thin outer layer. However, by increasing the ratio to 2, the inner part of the bead was also partially utilized for adsorption, and by increasing

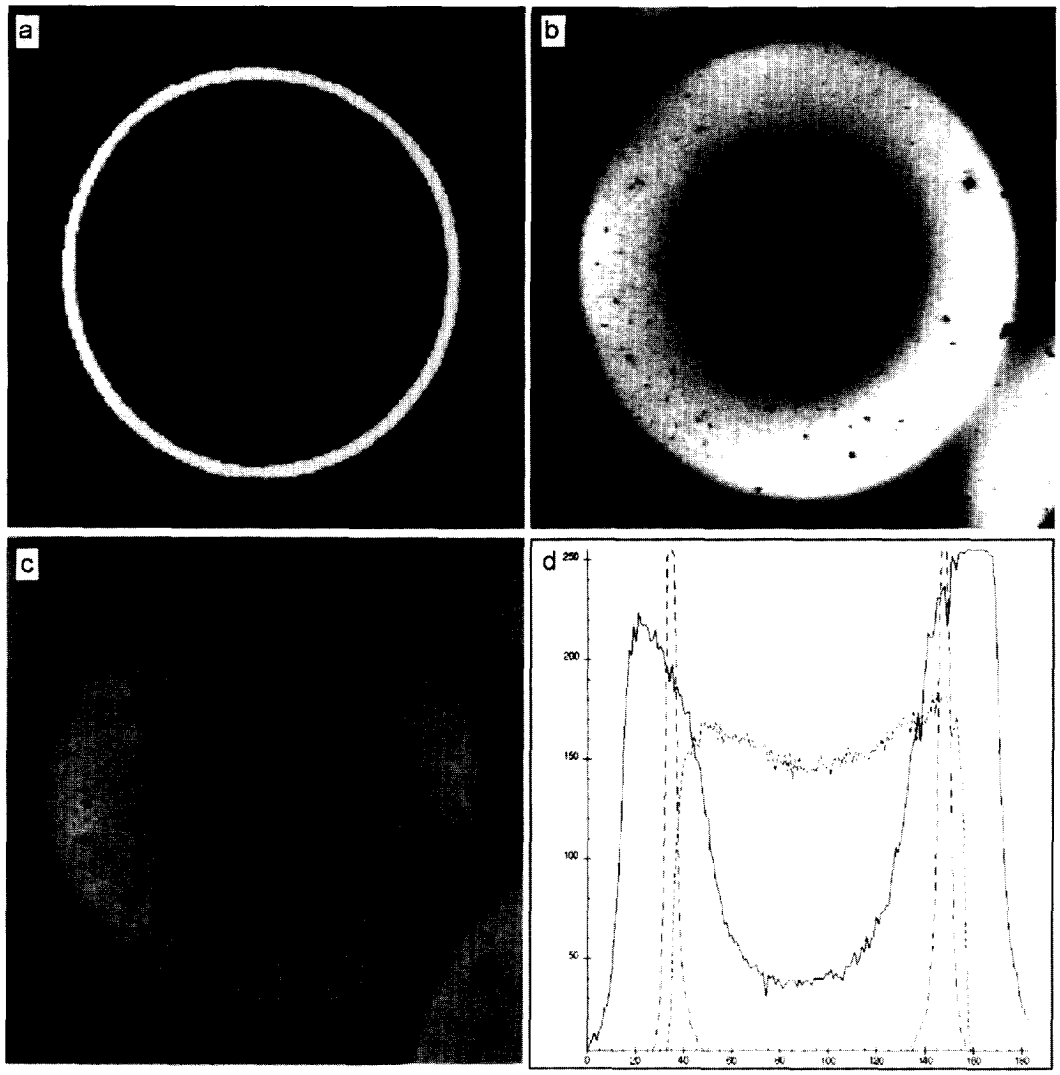

Fig. 5. Confocal image of a 50-μm particle. Incubation time, 1 min; laser, 0.4–1.4 mW, 488 nm; photomultiplier, 635–710 V. Section scan: Image size, 512×512 pixels; pixel size, 0.42 μm. (a) Sample–IgG Sepharose ratio=0.1. (b) Sample–IgG Sepharose ratio=2. (c) Sample–IgG Sepharose ratio=5. (d) Fluorescence intensity profile: x-axis shows pixels and y-axis shows the intensity (in arbitrary units), – – –=a, ···=b, ———=c.

the ratio to 5, Protein A was adsorbed throughout the whole bead.

Fig. 6a–d shows confocal images and fluorescence intensity profiles of 120 μm beads. In contrast to the experiments described above, the incubation Protein A and IgG Sepharose was allowed to continue for 10 min instead of 1 min. The result shows that by increasing the incubation time, the adsorption depth was increased also for the larger beads. Thus, a sample–IgG Sepharose ratio of 2 resulted in partial utilization of the inner parts of the bead, and at a ratio of 5 Protein A was adsorbed throughout the whole bead.

A stack of confocal images separated in space was used for three-dimensional reconstruction of the adsorption pattern in a 60-μm particle (Fig. 7a–f). The sample–IgG Sepharose ratio was 0.1 and the incubation time between Protein A and IgG Sepha-

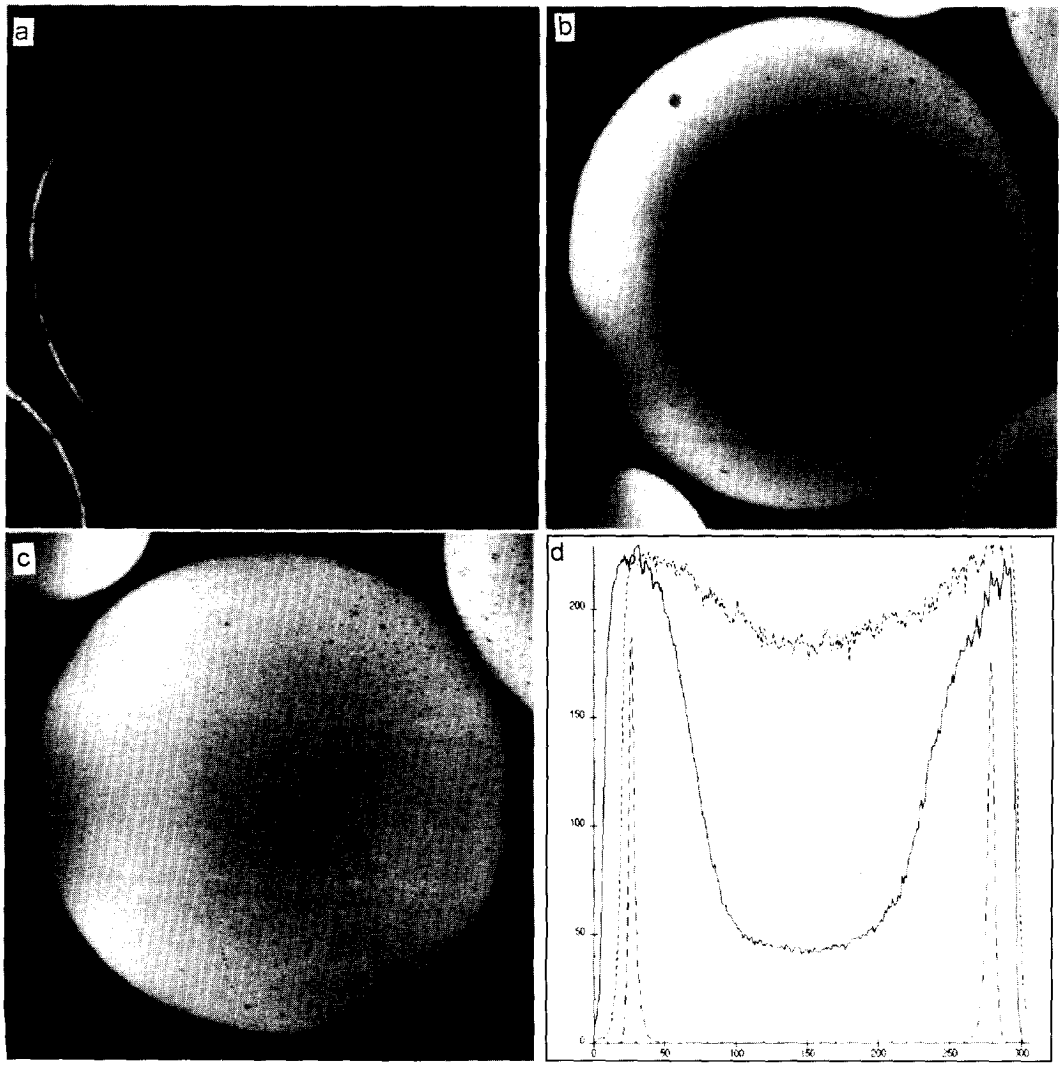

Fig. 6. Confocal image of a 120-μm particle. Incubation time, 10 min; laser, 0.4–1.2 mW, 488 nm; photomultiplier, 635–710 V. Section scan: Image size, 512×512 pixels; pixel size, 0.42 μm. (a) Sample–IgG Sepharose ratio=0.1. (b) Sample–IgG Sepharose ratio=2. (c) Sample–IgG Sepharose ratio=5. (d) Fluorescence intensity profile: x-axis shows pixels and y-axis shows the intensity (in arbitrary units), – – –=a, ———=b, ···=c.

rose was 1 min. As above, the result showed that Protein A had been adsorbed to an outer layer around the bead, while the interior of the bead was unused for adsorption. A problem in three-dimensional imaging is the darkening of the deeper layers, due to absorption and scattering of both the excitation and the fluorescent light, as mentioned above. Thus, the first image in the section series (Fig. 7a) ought to have the same intensity as the sixth image (Fig. 7f).

The results in this study show that optical sectioning by confocal microscopy is a powerful alternative technique to sectioning with a microtome. The technique, which is non-destructive, ought to be useful for studying both adsorption to and desorption from chromatographic matrices. Preliminary results show that the technique is useful not only for agarose-based matrices, but also for other types of gel media, for example, polymer-based ion ex-

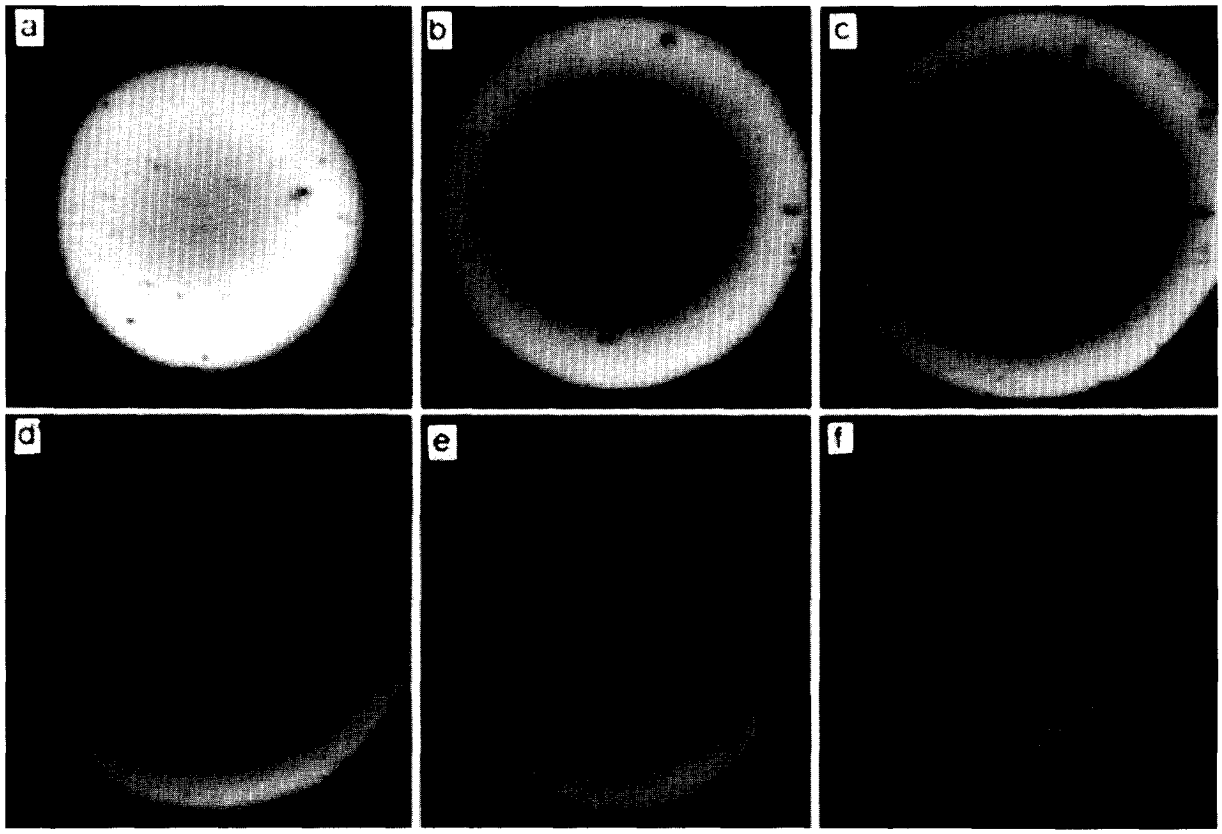

Fig. 7. Three-dimensional imaging of a 60-μm particle. The sample–IgG Sepharose ratio=0.1; incubation time, 1 min; laser, 1.5 mW, 488 nm; photomultiplier, 700 V. Section series: Image size, 256×256 pixels; pixel size, 0.9 μm. Scanning depth (a–f): ~5, 15, 25, 35, 45 and 55 μm, respectively.

changers like Mono Q (data not shown). Furthermore, a study of adsorption to single polymer-based cation exchanger particles using confocal microscopy and fluorescence intensity profile measurements has very recently been reported by Kim et al. [5].

The limitation of the method is set by the transparency of the particle, because of darkening of deeper layers due to absorption and light scattering.

4. Conclusions

The depth discriminating property of confocal microscopy can be used to obtain virtual optical slicing of a specimen. The technique, which is non-destructive, can be used as a tool for studying protein adsorption to chromatographic matrices.

Confocal scanning of individual IgG Sepharose beads was used for studying Protein A adsorption to IgG Sepharose 6 Fast Flow. The results showed that:

1. At low sample amounts, Protein A was adsorbed to a thin layer.
2. Immobilized IgG was distributed in the whole bead.
3. By increasing the sample–IgG Sepharose ratio, the adsorption depth also increased.
4. While the protein was adsorbed throughout the whole of a 50-μm particle, adsorption to larger beads decreased towards the center of the particle.

5. By increasing the incubation time, the adsorption depth was increased for larger beads.

A stack of confocal images separated in space was used for three-dimensional reconstruction of the adsorption pattern in a particle. A problem in three-dimensional imaging is the darkening of the deeper layers, due to absorption and scattering of both the excitation and the fluorescent light.

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