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Visualising intraparticle protein transport in porous adsorbents by confocal microscopy

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Abstract

Confocal scanning microscopy was used to study protein uptake to porous adsorbents during batch experiments in a finite bath. By coupling of a fluorescent dye to the protein molecules the penetration of single adsorbent particles at different times during batch uptake could be observed visually. Intensity profiles of the protein distribution within a single particle were obtained by horizontal scanning. After integration of the profiles the overall fluorescence within a bead could be calculated. Relating the overall fluorescence at different incubation times to the value at equilibrium allowed the construction of the fractional approach to equilibrium versus time. These data were compared to uptake curves, which had been obtained by measurements of the protein concentration in the supernatant and an excellent agreement of the curves was detected. The procedure was performed for two different proteins (lysozyme and human IgG) on two different media for protein adsorption (SP Sepharose Fast Flow and SP Sepharose XL; Pharmacia Biotech) and in all cases it could be shown, that the results from the direct measurements by confocal microscopy correspond very well to the data obtained from the indirect measurements in the fluid phase.

Keywords: Adsorption; Confocal scanning microscopy; Proteins; Finite bath adsorption; Protein diffusion

1. Introduction

The finite bath experiment is an efficient method for the determination of kinetics and equilibrium of protein adsorption. Models for evaluating these experiments in terms of intraparticle transport describe the fractional approach of the solid-phase protein concentration to equilibrium, i.e. Q/Q^{∞} as a function of t. The solid-phase concentration is in this case calculated from the reduction of the fluid phase protein concentration, assuming that each molecule, which disappears from the solution, is bound to the adsorbent. Thus only an indirect measurement of the protein concentration within the adsorbent is possible and a direct assessment of particle side concentration would be beneficial in order to supplement the data obtained from the indirect method via the fluid phase.

Recently, confocal scanning microscopy has been introduced as a tool for monitoring adsorption processes on the single particle scale. With the help of a confocal scanning microscope an optical sectioning of an adsorbent particle may be performed by observing the distribution of a fluorescent molecule within the particle. This technique was used by Kim et al. [1] in order to determine the uptake of a

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fluorescent dye into a polymeric ion-exchange resin. By measuring the concentration profile at different incubation times it was possible to show the slow diffusion of the dye within the resin. Ljunglöf and Hjorth measured internal concentration profiles of fluorescence labelled protein A within immunoaffinity adsorbent particles [2]. The varying depth of penetration of the protein into the porous beads under different experimental conditions was shown by horizontal scans, and an evaluation of the microscopic images yielded the fluorescence profiles across the particle diameter. A combination of both techniques together with a mathematical treatment of the data obtained may allow the construction of a batch protein uptake curve from a finite bath experiment based on a direct measurement of the solid-phase protein concentration.

The protein concentration in a single particle is supposed to be proportional to the overall fluorescence per unit particle volume, which can be obtained by integration of the intensity profile and by relating the integral intensity to the particle volume. If the integral intensity of a bead, which is in equilibrium with protein ($I^{\infty}_{\text{integr.}}$), is considered to represent the equilibrium capacity, then all other integral intensities at earlier times may be related to $I^{\infty}_{\text{integr.}}$ and a fractional approach to equilibrium is obtained without the necessity of calibrating the instrument for the actual protein concentration within the bead. Under this assumption the resulting curve may be plotted as Q/Q^{∞} versus t and may be evaluated according to standard models for finite bath uptake to porous particles. Additionally the protein concentration in the supernatant can be measured by absorbance at 280 nm and the solid-phase concentration can be calculated indirectly from the decrease in concentration according to Eq. (1).

$$Q = \frac{V_M(C_0 - C)}{V_S} \tag{1}$$

If both measurements produce the same uptake curve (Q/Q^{∞} vs. t), then a justification for using the decrease in fluid-side protein concentration as an indirect signal of the increase in solid-phase concentration during finite bath experiments is found. Furthermore a parity plot can be constructed from the integral fluorescence per particle volume, which is calculated from the confocal images, versus the capacity, which is calculated from the decrease in fluid-phase concentration.

In the present study the uptake of lysozyme and human Immunoglobulin G to two different media for protein adsorption will be analysed: SP Sepharose Fast Flow is a cation-exchange adsorbent for protein purification consisting of 6% crosslinked agarose, which provides a porous structure with an average pore radius of 24 nm [3]. SP Sepharose XL is a novel adsorbent, where an increase in static protein capacity is achieved by binding dextran spacers to the agarose backbone before coupling ion-exchange ligands. This layer of dextran spacers is supposed to increase the effective interacting volume.

2. Experimental

2.1. Instrumentation

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

2.2. Adsorbents, proteins, and process chemicals

SP Sepharose™ Fast Flow ($r_p = 3–7.5 \cdot 10^{-5}$ m) and SP Sepharose™ XL ($r_p = 3–7.5 \cdot 10^{-5}$ m) were obtained from Pharmacia Biotech (Uppsala, Sweden). Both commercially available adsorbents carry sulfopropyl-ligands with total ion-exchange capacity in the range of 180–220 mol H^+/m^3.

All chemicals were of analytical grade and were obtained from commercial sources. Lysozyme was purchased from Sigma–Aldrich (Stockholm, Sweden), polyclonal human Immunoglobulin (hIgG) was obtained from Pharmacia and Upjohn (Stockholm, Sweden) and Cy5 reactive dye from Amersham Life Science (Arlington Heights, USA). Labelling of the proteins with the fluorescence dye was performed according to the standard procedure recommended by the manufacturer.

2.3. Buffer systems

Lysozyme adsorption was performed in 50 m*M* glycin buffer at pH 9.0, whereas hIgG adsorption

was carried out in 50 m*M* sodium acetate buffer at pH 5.0.

2.4. Finite bath experiments

A 50-ml aliquot of protein solution was filled into a vessel equipped with a hanging stirrer. In order to minimise the consumption of fluorophor used per experiment, the labelled protein was mixed with unlabelled protein in a ratio of 1:20. A defined amount of adsorbent (typically 0.2–0.4 ml) was added to the respective buffer solutions resulting in a 1:1 slurry. The adsorption was started by adding the slurry to the stirred protein solution. At fixed times 0.5 ml of sample (fluid and adsorbent particles) was removed from the reaction vessel and quickly centrifuged at 14 000 *g* for 30 s. The supernatant was removed, stored for analysis of fluid phase protein concentration, and the sedimented particles were resuspended in 0.5 ml of the respective buffer solution (protein free). The centrifugation/resuspension cycle was repeated and followed by a final centrifugation, after which the supernatant was discarded. The washed adsorbent particles were transferred to the microscope for fluorescence analysis as described below. As control experiments protein adsorption to underivatised adsorbent (i.e. Sepharose 6 Fast Flow and dextran derivatised Sepharose 6 Fast Flow without ion-exchange ligands) was analysed. The background of fluorescence due to non-specific adsorption was found to be negligible (data not shown). Furthermore a comparison was made between confocal images, where all protein molecules were fluorescence labelled, and the standard conditions described above. As expected higher intensity values were obtained when undiluted solutions of labelled proteins were used. By adjusting the light intensity during excitation the resulting intensity profiles, however, were equivalent irrespective of dilution. Therefore a mixture of labelled and unlabelled protein in the ratio of 1:20 was used in this study.

2.5. Protein analysis

The protein concentration in the fluid phase was measured from the absorbance at 280 nm in a conventional UV spectrophotometer.

2.6. Confocal microscopic analysis

The basic principle of confocal imaging is that the specimen is illuminated one point at a time. The detector only registers fluorescent light reflected from that point, while light from out-of-focus planes is effectively blocked by a pinhole aperture in a mask positioned in front of the detector. This depth discriminating property can be used to slice the specimen optically into thin sections [4,5]. The basis of confocal scanning and its applications have been extensively described [6].

In this work individual beads were analysed by horizontal scanning (section scanning), i.e. the acquisition of two-dimensional confocal images perpendicular to the optical axis. A 60×1.4 objective lens was used for all measurements. The pinhole aperture was set to 50 μm. The argon/krypton laser provided excitation of Cy5 at 647 nm and emitted fluorescent light was detected at 660 nm. The image size for all images was 512×512 pixels[1] and the pixel size was 0.42 μm.

2.7. Evaluation of data from finite bath experiments

The scanning technique delivers an image of the distribution of fluorescent molecules in a horizontal section of an adsorbent particle. This picture is translated into a fluorescence intensity profile, which shows the fluorescence in arbitrary units as a function of the radial position within the particle expressed as pixel values . In order to transform the pixel values into a distance, the exact particle diameter is determined with help of the pixel size defined in the microscope's control software. In Fig. 1a a confocal image of an adsorbent particle, which was obtained after a horizontal scanning procedure, is shown. In Fig. 1b this image has been translated into a fluorescence intensity profile along the particle diameter. The overall fluorescence within the particle, which represents the amount of protein adsorbed, is calculated by dividing the particle radius into defined segments (equal to one pixel unit) and calculating the volume of a shell of the respective

[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.

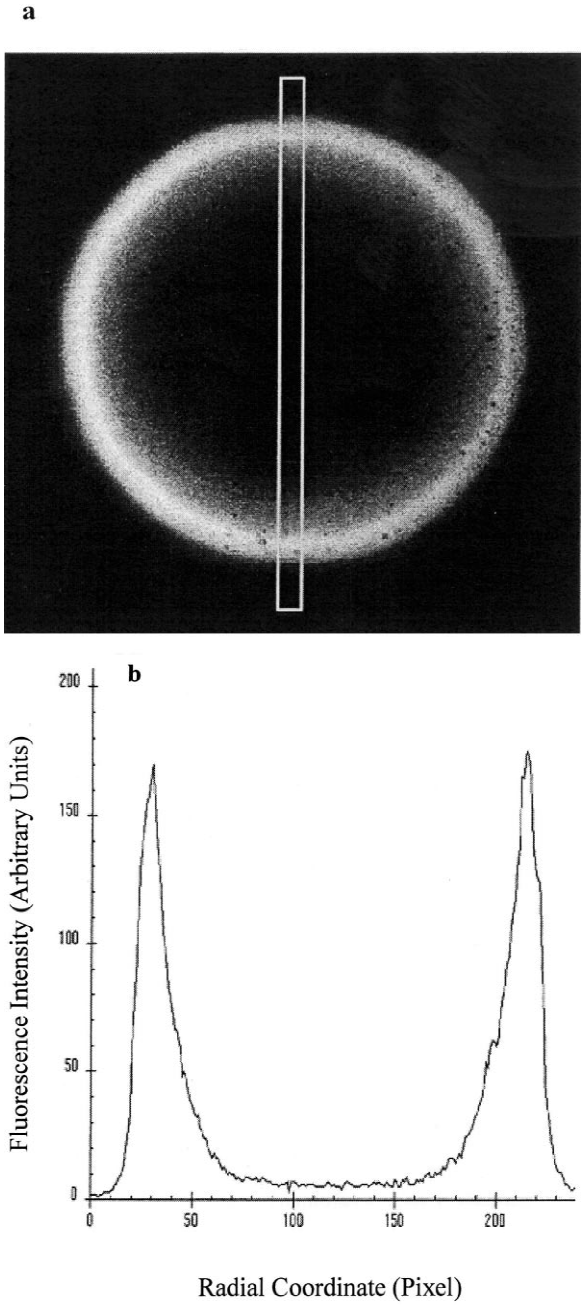

Fig. 1. (a) Confocal image of an adsorbent particle after incubation with fluorescence labelled protein in a finite bath experiment. The area, which was evaluated for the intensity profile is shown by the rectangular box. (b) Fluorescence intensity profile after image analysis (intensity in arbitrary units versus particle diameter in pixels).

thickness. Assuming that the average fluorescence within a segment represents the adsorbed protein concentration, we obtain the integral fluorescence within the shell, which stands for the amount of bound protein, from Eq. (2). The overall fluorescence per particle is calculated by summation over all individual shell elements. By relating this value to the particle volume the relative solid-phase concentration Q_{rel} is obtained in arbitrary units per m^3 of adsorbent (Eq. (3)).

$$I^{shell}_{integr.} = \bar{I}_{seg} \cdot [(r_a^3 - r_i^3) \cdot \tfrac{4}{3}\,\pi] \tag{2}$$

$$Q_{rel} = \frac{I_{integr.}}{V_P} = \frac{\sum_{shells} (I^{shell}_{integr.})}{V_P} \tag{3}$$

In order to construct the batch uptake curve the relative capacities are related to the relative capacity at equilibrium ($Q^{\infty}_{rel} = I^{\infty}_{integr.}/V_p$) and the saturation of the adsorbent with time is expressed as the fractional approach to equilibrium ($F = Q_{rel}/Q^{\infty}_{rel}$ versus t).

3. Results and discussion

As described in the previous section, finite bath experiments were performed with two different cation-exchange adsorbents and two different proteins. By taking samples of adsorbent particles at defined times and measuring the fluorescence profile across the beads, the progression of protein uptake with experimental time could be visualised. In Fig. 2 a series of scanning images for the adsorption of hIgG to SP Sepharose Fast Flow is shown. The adsorbent gradually fills with protein, as expressed by the penetration of fluorescing protein molecules into the internal regions of the beads. In Fig. 3 the respective translation into a series of fluorescence profiles is shown, where the fractional approach to equilibrium is shown by the increased area below the curves, which represents the amount of protein adsorbed. After integration and normalisation to the fluorescence profile at equilibrium the batch uptake curve is obtained as F versus t, which is shown in Fig. 4 for the system hIgG/SP Sepharose Fast Flow. Similarly the curves for hIgG/SP Sepharose XL (Fig. 5), lysozyme/SP Sepharose Fast Flow (Fig. 6),

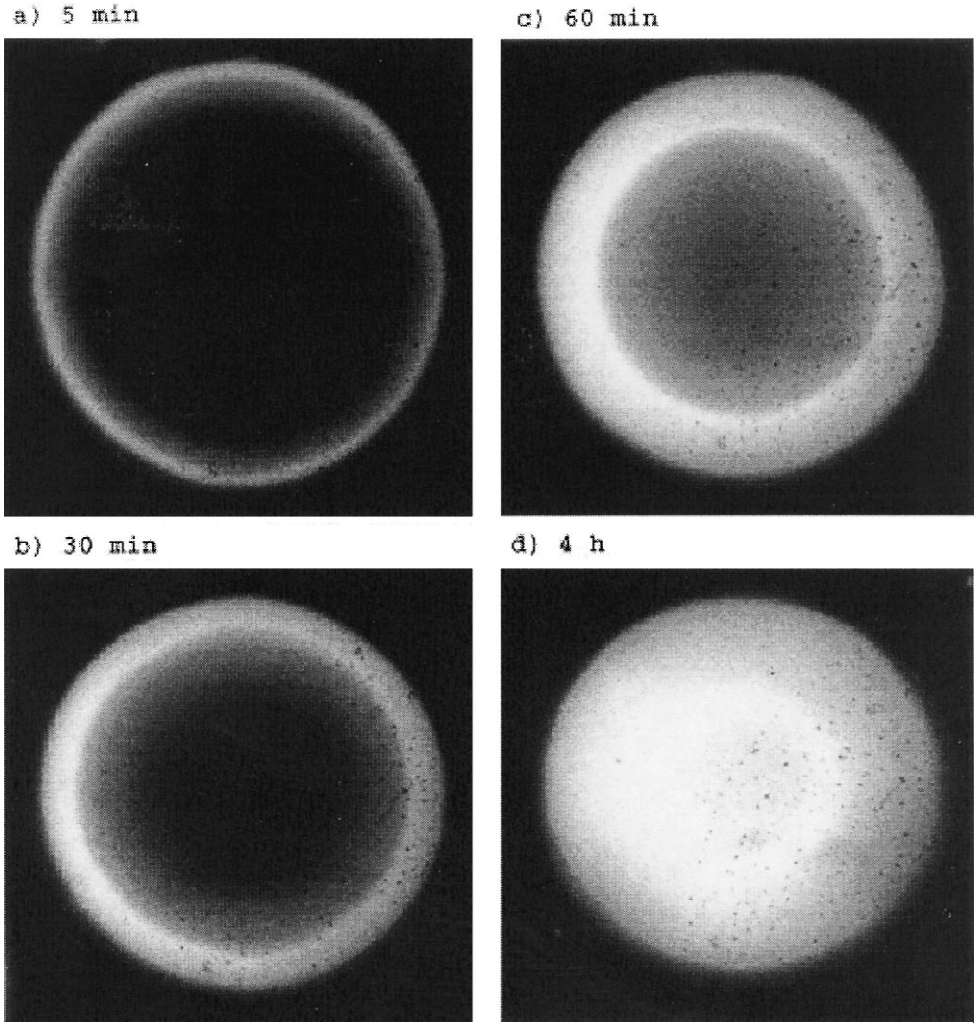

Fig. 2. Confocal images of samples from finite bath uptake of hIgG to SP Sepharose Fast Flow. (a) 5 min incubation time; (b) 30 min incubation time; (c) 60 min incubation time; (d) 240 min incubation time.

and lysozyme/SP Sepharose XL (Fig. 7) were created.

As it is important to cross-check the quantitative interpretation of the confocal images with an independent method, we wanted to correlate the direct measurement of protein uptake to the data obtained from indirect determination via the decrease in fluid phase protein concentration with time. For each sample, which was removed from the finite bath experiments for microscopic examination, we determined the protein concentration in the supernatant by absorbance at 280 nm. From Eq. (1) the increase in solid-phase concentration was calculated and plotted as the fractional approach to equilibrium over time. As an example of this procedure, the uptake of lysozyme by SP Sepharose XL is shown in Fig. 8. A comparison of the data (closed symbols) to the results from the microscopic analysis (open symbols) reveals a good agreement between the uptake profile from direct measurement and from indirect determination via the fluid phase concentration. It seems, however, that there is significantly more scatter in the data from the confocal microscope compared to the fluid phase data. This is not surprising, as we

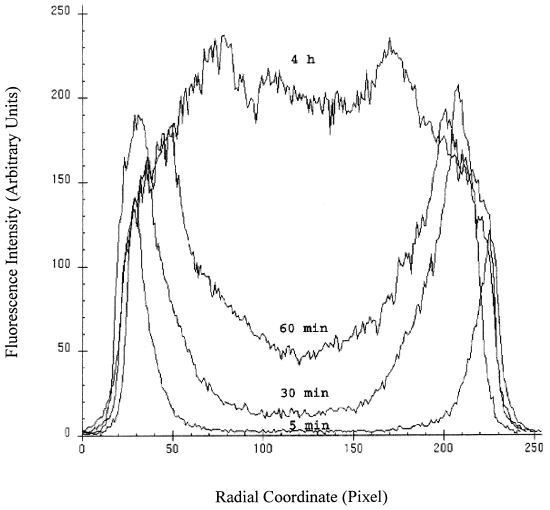

Fig. 3. Fluorescence intensity profile of the images in Fig. 2a–d.

measured fluorescence profiles of individual particles with the help of the confocal microscope, which were selected visually for similar size but still differed in diameter by approximately 1–5 μm. This difference in diffusive path length produces a slightly different dynamic uptake situation, where smaller particles will show more penetration relative to particles of larger diameter. Integrating the fluorescence profile will then translate the different uptake kinetics into a varying relative capacity, resulting in some variations in the plot of F versus t. In the fluid phase we measure the net result of protein uptake to all particles, so the result is an average over all particle diameters.

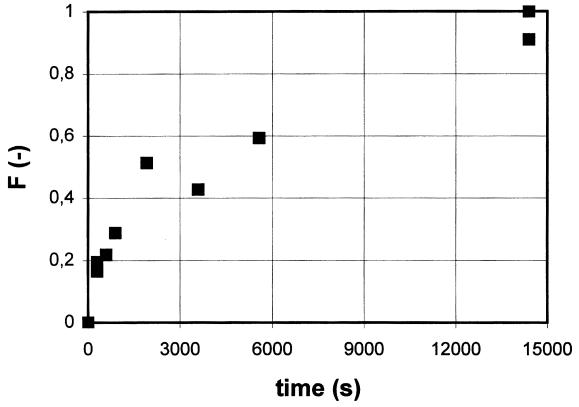

Fig. 5. Fractional approach to equilibrium during finite bath adsorption of hIgG to SP Sepharose XL.

In order to quantitatively examine the correlation between the direct measurements and the capacity calculated from the fluid phase we plotted the fluorescence intensity after integration Q_{rel} (Eq. (3)), which represents the overall protein concentration in the solid-phase, versus the capacity, which had been calculated from Eq. (1) from the respective fluid phase concentration. A linear relationship between directly and indirectly measured solid-phase concentration would support our assumption that the measurement of fluorescence profiles gives a realistic picture of the kinetics of protein uptake to porous

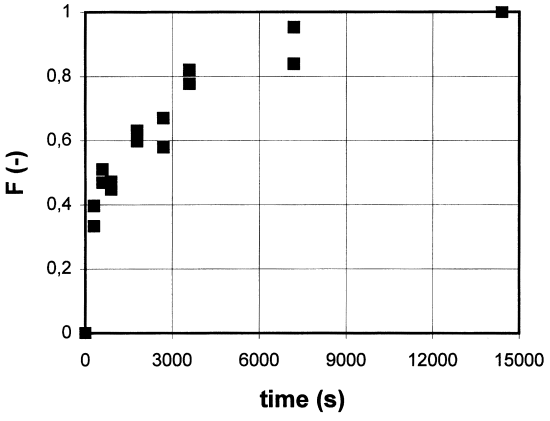

Fig. 4. Fractional approach to equilibrium during finite bath adsorption of hIgG to SP Sepharose Fast Flow.

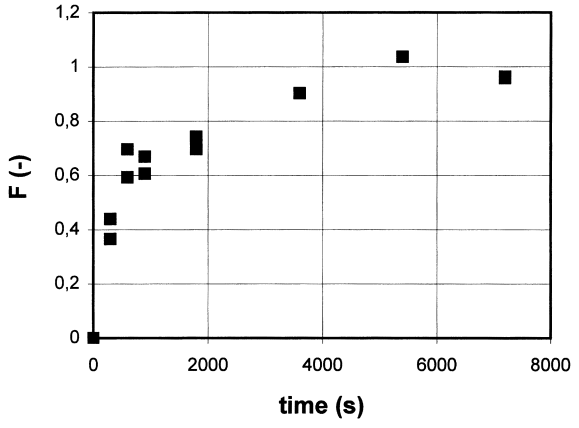

Fig. 6. Fractional approach to equilibrium during finite bath adsorption of lysozyme to SP Sepharose Fast Flow.

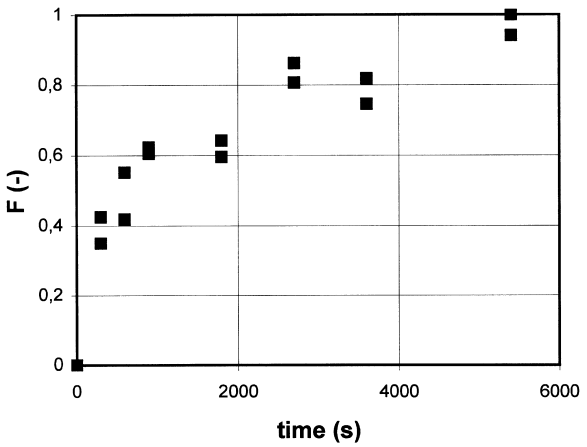

Fig. 7. Fractional approach to equilibrium during finite bath adsorption of lysozyme to SP Sepharose XL.

adsorbents. In Fig. 9 this plot for the four different experimental systems is shown. For each protein/adsorbent combination there is a good linear correlation, which may be taken as a validation of our approach. It is noteworthy that there is an individual correlation for each protein on different adsorbents, which is due to the fact that we were measuring the emission of fluorescence from immobilised protein molecules. As described by Liljeborg [7], both the laser light used for excitation as well as the emitted fluorescent light can be attenuated by absorption and scattering within the adsorbent bead. As there is a difference in chemical composition between the conventional porous media and the dextran derivatised media, it is not unexpected, that they differ in their optical properties and thus have an individual influence on the extent of light attenuation.

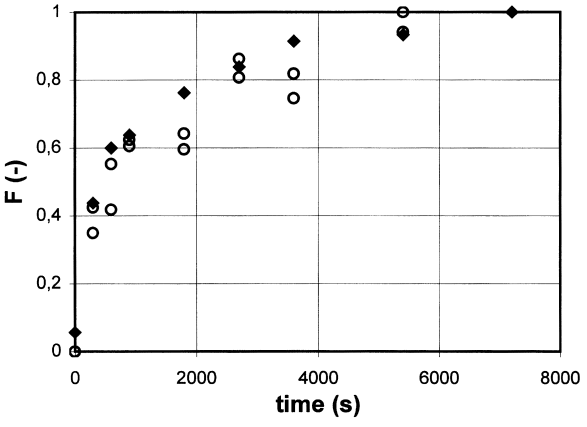

Fig. 8. Fractional approach to equilibrium for the adsorption of lysozyme to SP Sepharose XL. Open symbols – direct measurement with confocal scanning microscope, closed symbols – indirect measurement from fluid phase protein concentration.

4. Conclusions

Experimental data on equilibrium and kinetics of protein adsorption to porous particles mostly are determined by indirect measurements. The decrease in fluid phase concentration in finite bath experiments is recalculated in terms of a solid-phase concentration under the assumption, that each molecule, which vanishes from the fluid phase has been adsorbed to the solid phase. This is not an unrealistic assumption and a careful evaluation of finite bath uptake experiments delivers reliable estimations of parameters, e.g. equilibrium capacity or apparent internal diffusion coefficient, which can be used for the design of preparative scale adsorption processes. Nevertheless it would be desirable to measure the particle side protein concentration directly, in order to verify the assumptions. As confocal scanning microscopy had been shown to be a valuable method for the investigation of protein distribution within porous adsorbents in equilibrium situations, it was interesting to test the potential of this method for the evaluation of the dynamics of protein uptake. The data shown in an earlier paper [2] illustrated that the penetration of fluorescence marked proteins into porous particles can be translated into a fluorescence profile within the adsorbents. The data presented here demonstrated that the integration of this profile yields a total amount of fluorescence per unit particle volume, which correlates well with the solid-phase concentration calculated from the indirect determination via the fluid phase protein concentration. The development of the integrated fluorescence over time is a good representation of the fractional approach to equilibrium, which can be analysed mathematically by various methods [8,9] in order to obtain a quantitative description of the uptake kinetics.

This initial validation of the technology brings a variety of options for further use of confocal scanning microscopy. The velocity of protein influx into

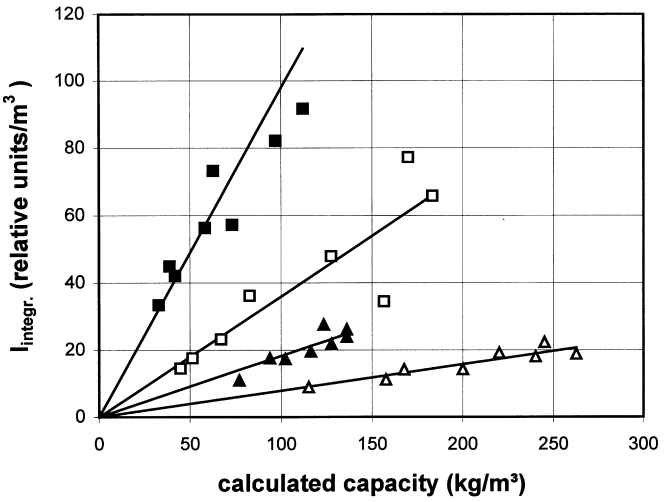

Fig. 9. Parity plot of Q_{rel} from integrated fluorescence profiles of confocal images per particle volume versus capacity calculated from decrease in fluid phase concentration according to Eq. (1). ■ = IgG/SP Sepharose Fast Flow; □ = IgG/SP Sepharose XL; ▲ = lysozyme/SP Sepharose Fast Flow; △ = lysozyme-SP Sepharose XL.

porous adsorbents is not only expressed by the development of the overall fluorescence within a particle with time, but the fluorescence profile itself is a direct function of intraparticle transport processes. Thus it may be used for an even more precise determination of the kinetics of protein adsorption. The simulation of protein uptake in a finite bath is based on the coupling of the fluid side and the particle side mass balance. A direct result of the solution of the particle side mass balance is the profile of the intraparticle protein concentration over the particle radius. The confocal scanning images contain an up to now unused potential for the comparison of experimental results with theoretical predictions of these profiles.

The confocal scanning microscope could not only be a valuable tool for the more theoretical work concerning protein adsorption, it may also be useful for the design of large scale adsorption processes. It is evident that this comparatively complex and expensive technology will not be suitable for the routine determination of equilibrium and kinetics of protein adsorption in order to obtain parameters for the design of process scale chromatography. It will, however, be a powerful method to validate experimentally simpler techniques, which can then be tested for their reliability and their potential for obtaining information on protein sorption. The data shown here were restricted to single component uptake from buffered solutions. It can be anticipated, however, that multi-component mixtures can also be evaluated by direct measurements using confocal scanning microscopy.

5. Symbols and abbreviations

C	protein concentration (kg/m^3)
C_0	initial protein concentration (kg/m^3)
F	fractional approach to equilibrium (-)
$I_{integr.}$	integral intensity of fluorescence profile (arbitrary units)
$I^{\infty}_{integr.}$	integral intensity of fluorescence profile at equilibrium (arbitrary units)
$I_{seg.}$	average intensity within a segment (arbitrary units)
Q	capacity (kg/m^3)
Q^{∞}	equilibrium capacity (kg/m^3)
Q_{rel}	relative capacity (arbitrary units/m^3)
Q^{∞}_{rel}	relative capacity at equilibrium (arbitrary units/m^3)
r_a	outer radius of particle shell (m)
r_i	inner radius of particle shell (m)
r_p	particle radius (m)
t	time (s)
V_M	fluid phase volume (m^3)

V_P particle volume (m^3)
V_S solid-phase volume (m^3)

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