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Direct visualisation of plasmid DNA in individual chromatography adsorbent particles by confocal scanning laser microscopy

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Abstract

Confocal microscopy was used for the measurement of plasmid DNA adsorbed to individual adsorbent particles intended for anion-exchange and triple helix affinity chromatography. Plasmid DNA was visualized with the fluorescent dye YOYO-1, that forms a highly fluorescent complex with double stranded DNA. Confocal images were translated into fluorescence intensity profiles and the distribution of plasmid DNA in the particles was measured. The results revealed that adsorption of plasmid DNA mainly takes place in an outer layer of the particles. The described procedure can also be advantageously used to demonstrate triple helix formation between plasmid DNA and immobilized oligonucleotides.

Keywords: Confocal scanning laser microscopy; DNA

1. Introduction

Chromatography is an essential technique in large scale purification of plasmid DNA, both as a process step and as an analytical tool. Different types of chromatography techniques such as reversed-phase [1], gel filtration [2], ion-exchange [3–5], and affinity [6–8] have been utilised. For large scale chromatographic purification of plasmid DNA, one of the major problems is the lower capacity of adsorbent particles as compared to protein purification. One important issue for developing better adsorbents for plasmid purification is knowledge about the distribution of DNA bound to the particles. The aim of this study was to evaluate the use of confocal microscopy for direct visualisation of plasmid DNA adsorbed to individual adsorbent particles.

In a confocal laser microscope, laser light is focused on one spot in the specimen. A fluorophore-stained sample in this spot emits fluorescent light that is focused again, and is allowed to pass through a pinhole aperture positioned in front of the detector. The aperture effectively blocks fluorescent light from out-of-focus planes. This depth discriminating property makes it possible to slice the specimen optically into thin sections [9,10]. By moving the focal point throughout the sample, the entire plane of interest can be scanned. Scanning in two dimensions at a given depth (i.e. horizontal scanning) provides a two-dimensional ‘optical section’ of the sample at that depth. The technique and its applications have been extensively described [11]. Among other applications, confocal scanning laser microscopy has been shown to be a useful tool for studying of protein

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adsorption on single chromatography adsorbent particles [12–14]. The technique has also been used for topographical imaging of macroporous microcarriers [15], measurement of three-dimensional distribution of DNA fragments within electrophoretic bands [16] and as a tool for morphological studies in polymer science [17].

Recently the characteristics of two novel high-affinity dyes, YOYO-1 [18] and PicoGreen [19] were described. Both dyes were reported to form highly fluorescent complexes with double stranded DNA, and the fluorescence enhancement upon binding was 500 and >1000, respectively. Visualising of DNA with YOYO-1 resulted in a considerably improved quality of fluorescence microscope images and was used for imaging of single DNA molecules [18].

In this study YOYO-1 was used to visualise plasmid DNA adsorbed to an anion exchanger (Q Sepharose XL) and a plasmid affinity media utilizing triple helix affinity [$(CTT)_7$ coupled to NHS-Sepharose High Performance]. Plasmid DNA was then detected by confocal imaging. Q Sepharose XL is a novel adsorbent, where dextran spacers are bound to the agarose backbone before coupling ion-exchange ligands. This layer of dextran spacers is supposed to increase the effective interacting volume, and results in an increased binding capacity. Triple helix affinity gels contain covalently bound oligonucleotides. These oligonucleotides can anneal to a complementary sequence introduced into the plasmid, forming a triple helix moiety [7].

2. Experimental

2.1. Instrumentation

Confocal microscopy analysis was carried out using a Multiprobe 2001 confocal scanning laser microscope supplied with an argon/krypton laser, and with ImageSpace software from Molecular Dynamics (CA, USA).

2.2. Adsorbents, plasmid DNA and chemicals

$(CTT)_7$ coupled to NHS-Sepharose High Performance for triple helix affinity chromatography (below named THAC-media) was prepared as described in Ref. [8]. The oligonucleotide $(CTT)_7$ was synthesised using OligoPilot System (Amersham Pharmacia Biotech) with a NH_2 group introduced at the 5′-end. The oligonucleotide had a purity of ≈80% when analysed on capillary electrophoresis and reversed-phase chromatography. NHS-Sepharose High Performance and Q Sepharose XL were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Plasmid DNA, 6.3 kilo base pairs (kbp) in size, with insert sequence $(GAA)_{17}\cdot(CTT)_{17}$, was kindly gifted from Rhone Poulenc Rorer, France. YOYO-1 in 1 m*M* DMSO (Y-3601) was obtained from Molecular Probes Europe (Leiden, The Netherlands). All other chemicals were of analytical grade and were obtained from commercial sources.

2.3. Staining of plasmid DNA adsorbed to chromatography adsorbent particles

2.3.1. THAC-media

A 0.5-ml volume of THAC-media in 1 ml 0.2 *M* sodium acetate, 2 *M* NaCl, pH 4.5 (buffer A) was incubated end-over-end overnight with 150 μl plasmid (252 μg in 10 m*M* Tris, 1 m*M* EDTA pH 8)+150 μl 0.4 *M* sodium acetate, 4 *M* NaCl, pH 4.5, giving a final concentration of 194 μg/ml (504 μg/ml gel). This prototype gel had a binding capacity of around 200 μg plasmid/ml gel. The relatively long incubation time was to ensure that total equilibrium was reached, and is not related to exposure times used in preparative chromatography. Control experiments were also performed with NHS-Sepharose without oligonucleotide, and with blanks, where 150 μl 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8 was added instead of the plasmid sample. However, plasmid DNA and YOYO-1 were not incubated together, as binding of the positively charged dye [18] to the plasmids might affect the adsorption.

After incubation, the particles were washed with 4×2 ml buffer A to remove any unbound plasmid, and finally a 50% slurry was prepared. Furthermore, end-over-end incubation of 100 μl of the 50% slurry and 100 μl 6 μ*M* YOYO-1 (diluted in buffer A) was performed for 60 min. Individual particles were then measured with confocal microscopy and analysed as described below.

2.3.2. Q Sepharose XL

A 100-μl volume of media was incubated overnight with 1.4 ml of plasmid (135 μg/ml) in 0.5 *M* NaCl, 25 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0 (buffer B). The unbound plasmid was washed away with 4×1 ml of the same buffer. The concentration of adsorbed plasmid on the gel was 910 μg/ml gel. Further staining with YOYO-1 (diluted in buffer B) was performed as described above.

2.4. Confocal microscopy

Individual adsorbent particles were analysed by acquisition of two-dimensional confocal images perpendicular to the optical axis, i.e. horizontal scanning. A 60×1.4 oil immersion objective[1] was used for all measurements and the pinhole aperture was set to 50 μm. The laser provided excitation of YOYO-1 at 488 nm and emitted fluorescent light was detected at 510 nm. The laser intensity was between 1 and 5 mW and the photomultiplier voltage was 700 V. The image size for all images was 512×512 pixels. The pixel size was 0.42 μm, where a pixel is defined as a two-dimensional picture element in a confocal image and the pixel size is related to the distance between scanned points.

2.5. Evaluation of confocal images

The distribution of fluorescent molecules through the adsorbent particles was obtained by translation of confocal images into fluorescence intensity profiles, as earlier described [13,14]. The intensity profiles, shows the fluorescence in arbitrary units as a function of the radial position within the particle expressed as pixel values. The thickness of the adsorption layer was measured at the base of the triangle obtained by drawing the tangents to the intensity peak edges (Fig. 1).

[1]The optical depth resolution (R_d) obtainable in confocal microscopy using different microscope lenses (N.A.≤1.4) can be approximated by $R_d = (1.4\ n\lambda)/(\text{N.A.})^2$ where N.A. is the numerical aperture of the microscope objective in use, λ is the wavelength of the detected light and n is the refractive index of the mount medium [20]. The set-up in this study gives a theoretical depth resolution of 0.55 μm.

2.6. Frontal analysis

Plasmid DNA (0.1 mg/ml dissolved in 25 m*M* Tris–HCl+0.5 *M* NaCl, pH 8.0) was applied on 0.5×5 cm columns packed with Q Sepharose XL, at a fluid velocity of 100 cm/h (0.33 ml/min), until the UV signal (C/C_0) reached >5%.

The breakthrough capacity, here defined as the amount of plasmid DNA which has been adsorbed to the ion exchanger when the effluent concentration, C, reaches 5% of the initial sample concentration C_0, was calculated according to Eq. (1)

$$Q_{B,5\%} = (V_{5\%} - V_0)C_0/V_c \quad (1)$$

where $Q_{B,\,5\%}$ = mg plasmid/ml adsorbent at 5% breakthrough, $V_{5\%}$ = applied volume of sample at 5% breakthrough, C_0 = sample concentration (mg/ml), V_c = geometric total volume and V_0 = void volume.

2.7. Calculation of available surface area

Calculation and comparison of the available surface area in a column packed with Q Sepharose XL (particle size 45–165 μm; volume median diameter, x_{50} = 90 μm) and a sieved fraction of the same adsorbent (50–60 μm; x_{50} = 55 μm) was made using Eqs. (2–5). In this way the available surface area for Q Sepharose XL was estimated to be $4.7\cdot10^{-2}$ m^2/ml packed bed and $7.6\cdot10^{-2}$ m^2/ml for the sieved fraction. Thus, a ≈1.6-fold increase was achieved with the sieved fraction.

$$V_{particle} = \frac{4}{3}\pi r^3 \quad (2)$$

$$N_{particle} = V_c\,(1-\varepsilon)/V_{particle} \quad (3)$$

$$A_{particle} = 4\pi r^2 \quad (4)$$

$$A_{particle,\,tot} = A_{particle} \times N_{particle} \quad (5)$$

where $V_{particle}$ = volume of a particle, r = particle radius, $N_{particle}$ = number of particles in a column, V_c = geometric total volume, ε = interparticle porosity, $A_{particle}$ = surface area of a particle and $A_{particle,\,tot}$ = total available surface area in the column.

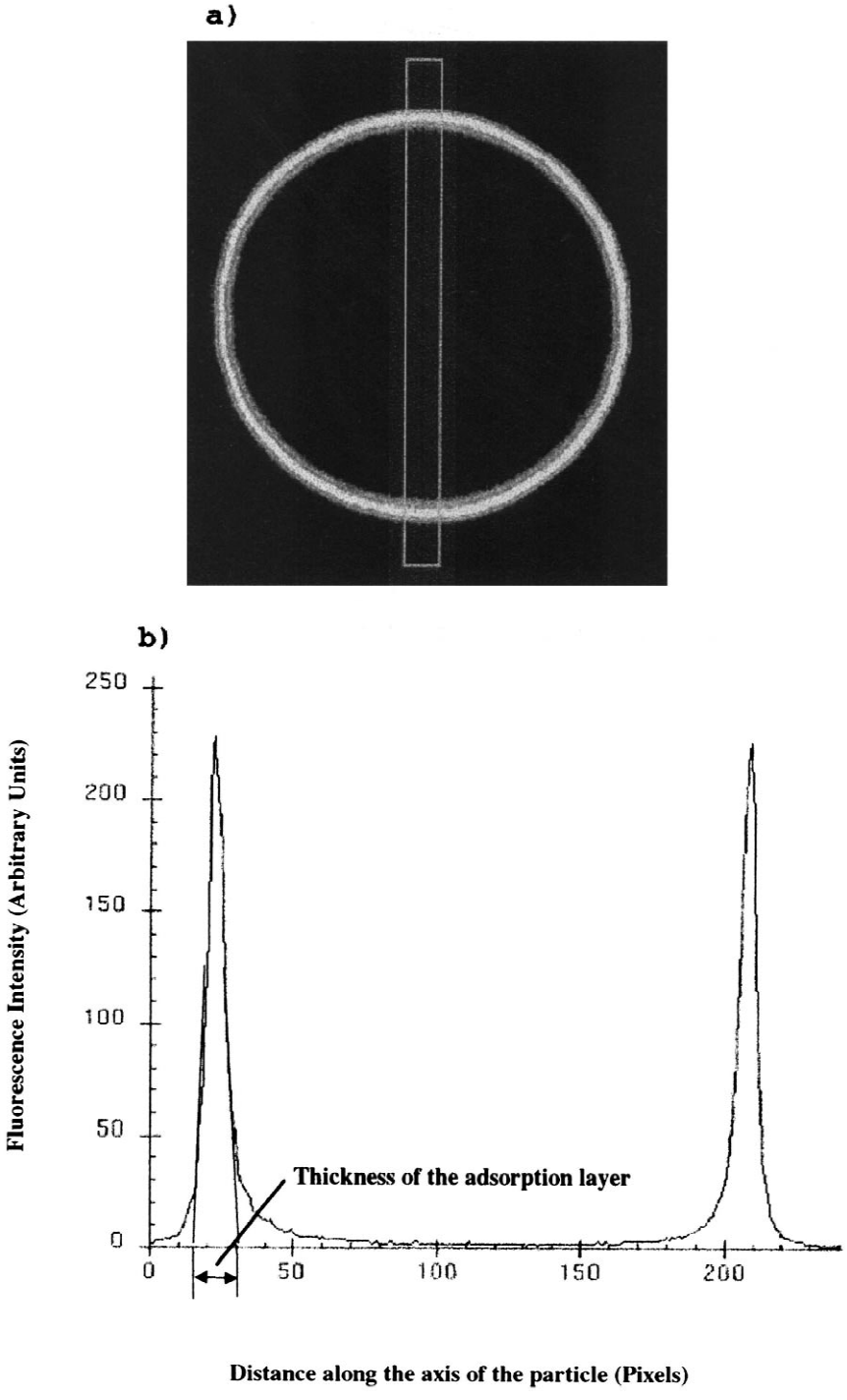

Fig. 1. Translation of a confocal image into a fluorescence intensity profile. (a) Confocal image of Q Sepharose XL saturated with plasmid DNA. The rectangular box shows the area that was evaluated. (b) Fluorescence intensity profile after image analysis: *x*-axis shows distance along the axis of the particle (in pixels of 0.42 μm) and *y*-axis fluorescence intensity (in arbitrary units).

Table 1
Experimental combinations of chromatography media, plasmid DNA and YOYO-1

No.	Adsorbent	Plasmid DNA	Yoyo-1	Result
1	NHS-Sepharose HP	−	−	No signal
2	NHS-Sepharose HP	−	+	No signal
3	NHS-Sepharose HP	+	+	No signal
4	THAC-media	−	+	≈33× weaker signal than no. 5
5	THAC-media	+	+	Strong signal in an outer shell.

3. Results

Different combinations of chromatography media (THAC media) with and without oligonucleotide, plasmid DNA and fluorophore were examined, according to Table 1. No signal could be detected from particles without immobilised oligonucleotide. However, the combination of immobilised oligonucleo-

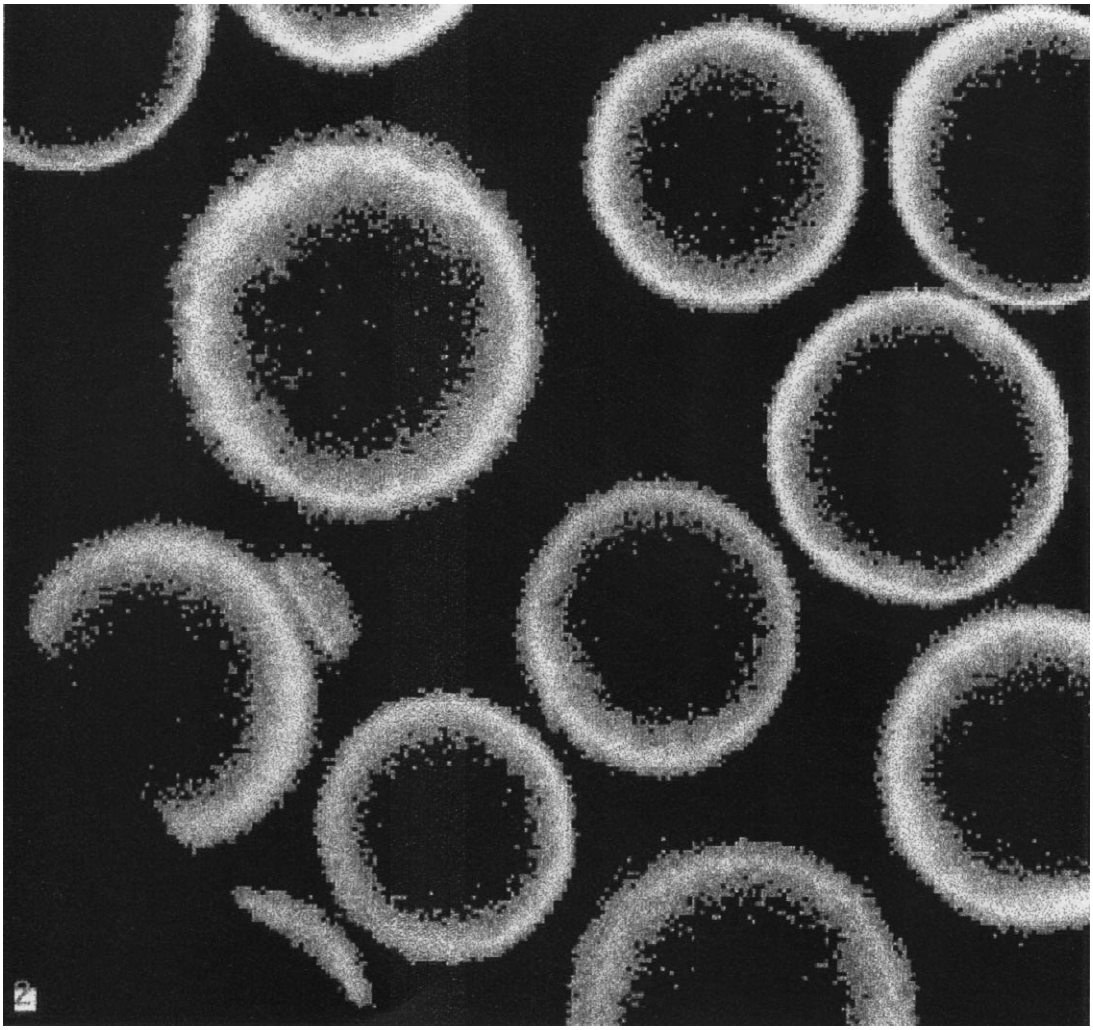

Fig. 2. Confocal images of THAC-media after saturation with plasmid DNA and visualisation with YOYO-1.

Table 2
Thickness of the adsorption layer of plasmid DNA on THAC media and Q Sepharose XL

Adsorbent	Particle size (μm)	Adsorption layer (μm)
THAC media[a]	25	3.0
	26	3.0
	30	3.0
	34	3.3
Q Sepharose XL[b]	65	6.1
	88	6.4
	111	7.3

[a] Particle size for NHS-Sepharose HP is 34±10 μm.
[b] Particle size for Q Sepharose XL is 45–165 μm (mean particle size 90 μm).

tide and plasmid DNA visualised with YOYO-1 gave a strong signal in a thin outer layer of the particles (Fig. 2), while absence of plasmid gave a weak background signal of about 3%. In the same way different combinations of Q Sepharose XL, oligonucleotide or plasmid DNA and YOYO-1 were tested. The result reveals that a signal is obtained from double stranded plasmid DNA adsorbed to the particles, while no signal is obtained from single stranded oligonucleotide or from the particles themselves.

In an additional series of experiments, THAC media and Q Sepharose XL were saturated with plasmid DNA, visualised by YOYO-1 and analysed by confocal scanning as above. The confocal images were translated into fluorescence intensity profiles and the thickness of the adsorption layer was measured. The results (Table 2) show that adsorption of plasmid DNA mainly takes place in an outer layer, while the interior of the particles remains empty. Thus, a smaller particle diameter will result in an increased proportion of outer surface area, and therefore in a higher binding capacity.

To confirm this conclusion, a control experiment was performed where a comparison was made between commercially available Q Sepharose XL (particle size 45–165 μm) and a sieved fraction of the same adsorbent with a narrower particle size distribution (50–60 μm). Frontal analysis was used to determine the breakthrough capacity. The result (Table 3) shows that the breakthrough capacity ($Q_{B,5\%}$) increased 1.56 times on the sieved fraction, which corresponds well with the increase in available surface area that was estimated at 1.6 (see Section 2.7).

4. Discussion

The present work demonstrates the strength of confocal imaging for measurement of plasmid DNA adsorbed to individual adsorbent particles. After visualisation of DNA with a fluorescent dye and translation of the confocal images into fluorescence intensity profiles, the distribution of plasmid DNA in the particles can be measured. By visualisation of DNA with a dye (like YOYO-1) that forms a fluorescent complex with double stranded DNA, but not with single stranded DNA, the procedure can also be used to demonstrate binding between plasmid DNA and immobilised oligonucleotides, and consequently for evaluation of various oligonucleotides as triple helix affinity ligands. As confocal microscopy enables measurement in individual particles, only small amounts of plasmid DNA and oligonucleotides are required.

The confocal images reveal that adsorption of plasmid DNA to THAC media and Q Sepharose XL mainly takes place in an outer layer of the particles. This observation agrees well with observations by Prazers et al. [4], that anticipated that only a small fraction of the internal void volume of polystyrene–divinyl benzene and agarose based resins are accessible to the long and thin plasmid molecules. As a consequence, the majority of the binding will occur

Table 3
Comparison of breakthrough capacity at 5% breakthrough ($Q_{B,5\%}$)

Adsorbent	Particle size (μm)	Plasmid (mg/ml adsorbent)
Q Sepharose XL	45–165	3.4
Q Sepharose XL (sieved fraction)	50–60	5.3

on the outer surface of the particles. However, by decreasing the particle size, the outer surface area available for adsorption is increased. This in turn results in an increased binding capacity, expressed as mg plasmid/ml adsorbent. Thus, for comparison of different adsorbents intended for plasmid purification, it is important to consider that the particle size has an essential influence on the capacity.

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