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# Simultaneous characterization of ligand binding properties and aggregation state of proteins using affinity CE combined with Taylor dispersion analysis

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## Introduction

Characterization of proteins and their interactions is fundamental to the understanding of biochemical processes since most biomolecular processes involve the molecular recognition and ligand binding by proteins. Protein based drugs represent special challenges in terms of drug development and manufacturing. The structural integrity is sensitive to the conditions encountered during formulation, production and storage. The therapeutic efficacy is closely related to the conformational structure; thus development of protein drugs requires a detailed knowledge of the physical and chemical stability. In this context, it would be beneficial if biophysical and functional characterization could be combined into one method.

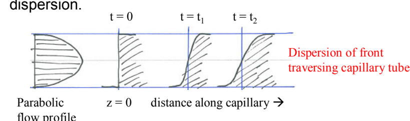
## Aim

To develop a CE based method suitable for simultaneous characterization of protein function, in the form of ligand binding, and determination of protein aggregation state through size measurements.

To combine affinity CE in frontal analysis mode, widely used in interaction studies, with Taylor dispersion analysis (TDA) used for measurement of diffusion coefficients (hydrodynamic radii; sizing).

### Taylor dispersion[1-3]

The velocity of laminar flow through a capillary vary over the capillary cross-section according to a parabolic function. Thus analytes move with different velocities according to the position in the capillary cross-section. Diffusion occurs over the capillary cross-section as well as along the capillary axis. The combined action of molecular diffusion and convection lead to a special form of dispersion termed Taylor dispersion.




### Instrumentation

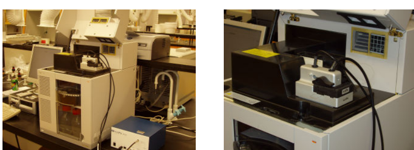


Figure 1 Agilent 3DCE combined with Paraytec's Actipix D100 UV area imaging detector. Actipix sensor head is placed under CE cover during analysis.

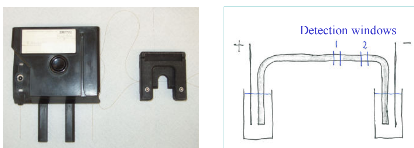



Figure 2 Capillary looping from Agilent cassette into Actipix CE cartridge and back allowing two detection points along the capillary.

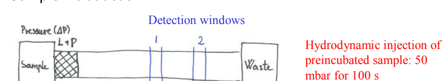
## Experimental

**Solutions and samples.** 67 mM sodium phosphate buffer, pH 7.40 was used as electrophoresis and sample buffer. Samples containing $5.0 \cdot 10^{-5}$ M propranolol and $2.5 \cdot 10^{-5}$ M $\alpha_1$-acid glycoprotein (AGP) or $8.0 \cdot 10^{-5}$ M human serum albumin (HSA) were prepared in the phosphate buffer (pH 7.4). Standards contained $5.0 \cdot 10^{-5}$ M propranolol.

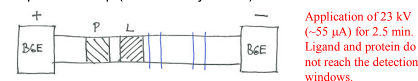
**CE** was performed on an Agilent 3DCE coupled to an Actipix D100 UV area imager (Paraytec Ltd, York, UK). Uncoated fused silica capillaries were 92 cm in total length (50 μm i.d.) and lengths of 34.5 and 49 cm to the first and second detection window, respectively. Detection at 200 nm; Capillary cassette temperature 25°C; Rinse: 0.1 M NaOH and buffer for 3 min each.

### Frontal analysis CE – TDA procedure

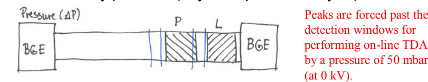
Sample introduction:




Separation step (frontal analysis CE):




Mobilisation by pressure (Taylor dispersion analysis):




## Results

Frontal analysis conditions (presence of plateau peaks) were achieved with an injection time of 100 s (50 mbar) corresponding to ~94 nL sample volume (Figure 3).

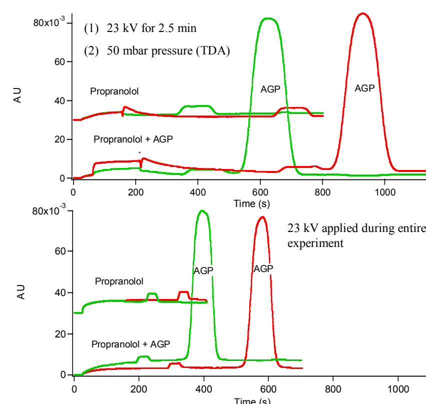



Figure 3 Frontal analysis CE with (upper traces) and without pressure mobilisation (lower traces). Shifted traces are propranolol standards used in the determination of the degree of ligand binding.

### Taylor dispersion analysis

Due to the large sample volumes protein peaks were fitted to TDA expression for fronts (Eq 1) (Figure 4). Temporal variances were obtained for both the advancing and trailing fronts for AGP.

$$\frac{C}{C_0} = \frac{1}{2} \pm \frac{1}{2}\,\mathrm{erf}\left(\frac{(t - t_R)}{\sigma\sqrt{2}}\right) \quad \text{Eq 1}$$

$$D = \frac{R_C^2 (t_2 - t_1)}{24(\sigma_2^2 - \sigma_1^2)} \quad \text{Eq 2}$$

$$D = \frac{k_B T}{6\pi\eta R_h} \quad \text{Eq 3}$$

Diffusion coefficients were obtained from the change in front sharpness (temporal variance) from window 1 to 2 (Eq 2) and hydrodynamic radii from Eq 3.

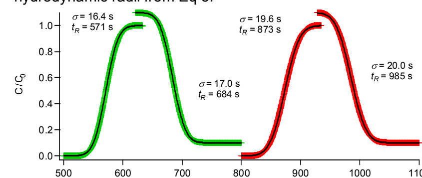



Figure 4 TDA (Eq 1) performed on normalised traces from Fig 3

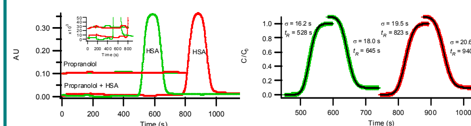



Figure 5 Frontal analysis CE followed by pressure mobilisation for propranolol-HSA sample. Left: TDA (Eq 1) performed on normalised traces

### Frontal analysis CE – TDA data

**Table 1. Fraction of free propranolol ($f_{free}$), $\alpha_1$-acid glycoprotein (AGP) and human serum albumin (HSA) diffusion coefficients (*D*) and Stokes hydrodynamic radii ($R_h$) obtained using frontal analysis CE and Taylor dispersion analysis in 0.067 M phosphate buffer (pH 7.40) at 25°C (Mean ± SD; n = 3).**

| Sample | $f_{free}$ | $10^{11} \cdot D$ ($m^2\,s^{-1}$) | $R_h$ (nm) | $R_h$ litt. (nm) |
|---|---|---|---|---|
| Propranolol -AGP[a] | $0.66 \pm 0.02$ | $6.89 \pm 0.14^{d}$ | $3.56 \pm 0.07^{d}$ | 3.2[4];3.3[5]; 3.5[5] |
| Propranolol -AGP[b] | $0.64 \pm 0.003^{c}$ | - | - | |
| AGP[a] | - | $6.67 \pm 0.15^{d}$ | $3.63 \pm 0.08^{d}$ | |
| Propranolol -HSA[a] | $0.87 \pm 0.001^{c}$ | $6.59 \pm 0.12^{e}$ | $3.73 \pm 0.07^{e}$ | 3.3–4.1[6]; 3.4-3.6[7] |
| Propranolol -HSA[b] | $0.95 \pm 0.002^{c}$ | - | - | |
| HSA[a] | - | $6.41 \pm 0.18^{e}$ | $3.83 \pm 0.11^{e}$ | |

*a* Pressure applied; combined CE and TDA
*b* Pressure not applied; conventional frontal analysis CE
*c* AGP, HSA and propranolol concentrations were $5.0 \cdot 10^{-5}$ M, $8.0 \cdot 10^{-5}$ M and $2.5 \cdot 10^{-5}$ M, respectively
*d* Advancing and trailing fronts used for determination of *D* and $R_h$ (n = 2 × 3)
*e* Advancing fronts only used for determination of *D* and $R_h$ (n = 3).

Binding data obtained be the combined CE-TDA method is in accordance with results from traditional frontal analysis CE. Propranolol binding results of AGP and HSA are in qualitative agreement with literature reports (higher affinity of propranolol for AGP than for HSA).
Hydrodynamic radii (sizing) results are consistent with previously published results for the proteins.

## Conclusion

- From two CE runs (sample and ligand reference) protein function can be characterized in form of ligand binding ability and protein hydrodynamic radius determined from on-line measurement of diffusion coefficients.
- The UV area imaging detector allowing two detection points facilitates combination of frontal analysis CE and Taylor dispersion analysis and reduces the number of experiments needed for characterization of protein size and ligand binding properties.
- Results obtained in form of hydrodynamic radii and degree of binding are in agreement with literature values for the model affinity systems investigated.
- The methodology is expected to be of interest for characterization of pharmaceutical proteins by providing information related to protein aggregation state (presence of monomer, dimers and oligomers) in addition to functional protein characterization.

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### References

(1) Aris. *Proc. R. Soc. London, Ser. A* **1956**, *235*, 67-77. (2) Taylor. *Proc. R. Soc. London, Ser. A* **1953**, *219*, 186-203. (3) Sharma *et al*. *Anal. Chem.* **2005**, *77*, 806-813. (4) Bezkorovainy, A. *Biochim. Biophys. Acta* **1965**, *101*, 336-342. (5) Kawahara *et al*. *Biochim. Biophys. Acta* **1973**, *295*, 505-513. (6) Jachimska *et al*. *Langmuir* **2008**, *24*, 6866-6872. (7) Aragon & Hahn. *Biophys. J.* **2006**, *91*, 1591-1603.