ELSEVIER

Journal of Chromatography A, 844 (1999) 395–401

JOURNAL OF CHROMATOGRAPHY A

Short communication

Reassessment of the calibration constant for the IAsys biosensor

Damien R. Hall, Donald J. Winzor*

Centre for Protein Structure, Function and Engineering, Department of Biochemistry, University of Queensland, Brisbane, Queensland 4072, Australia

Received 1 December 1998; received in revised form 9 March 1999; accepted 9 March 1999

Abstract

A magnitude of 50 arc s ng^{-1} mm^2 has been determined for the calibration constant relating biosensor response to the amount of protein bound to the sensor surface of an IAsys cuvette. These studies entailed enzymatic assessment of the extent of lactate dehydrogenase depletion in the liquid phase arising from enzyme binding to a carboxymethyldextran-coated sensor surface, and also estimation of a maximum biosensor response for the electrostatic interaction of ovalbumin with an aminosilane-coated sensor surface. The latter results required correction for contributions to biosensor response resulting from changes in the refractive index of the liquid phase effected by high protein concentrations. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Biosensors; IAsys calibration; Calibration; Ligate depletion; Detection, LC; Proteins

1. Introduction

Because of the cuvette-based design of the IAsys biosensor [1], the binding of ligate to affinity sites immobilized on the sensor surface occurs at the expense of the ligate concentration in the liquid phase. Although this effect was neglected initially [2–4], the importance of making allowance for ligate depletion in the liquid phase has now been recognised [5–8]. At present the extent of depletion is calculated on the basis of a calibration constant of either 163 arc s ng^{-1} mm^2 [2] or 206 arc s ng^{-1} mm^2 [3] for the instrumental response – values obtained with ^{125}I-labelled human serum albumin as a tracer to relate optical response to the amount of albumin bound to anti-albumin immunoglobulin G (IgG) immobilised on the sensor surface [2,3]. A potential deficiency of that approach is its reliance upon an assumption that the matrix-bound monoclonal antibody exhibits equal affinities for its eliciting antigen and the iodine-modified derivative(s) thereof. Alternative procedures for measuring the calibration constant have therefore been devised to determine its magnitude, which clearly has a direct influence on the calculated extent of ligate depletion in the liquid phase for each measured instrumental response in either kinetic or thermodynamic characterisation of the ligate–matrix interaction.

2. Experimental

2.1. Materials

Crystalline preparations of bovine serum albumin, ovalbumin and rabbit muscle lactate dehydrogenase

*Corresponding author. Fax: +61-7-3365-2132.
E-mail address: winzor@biosci.uq.edu.au (D.J. Winzor)

0021-9673/99/$ – see front matter © 1999 Published by Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00357-X

were obtained from Sigma (St. Louis, MO, USA). All chemicals were of reagent grade.

2.2. Preparation of solutions

Stock solutions of the three proteins were prepared by exhaustive dialysis against the solvent to be used in the relevant IAsys experiment, the final diffusate being used to equilibrate and fill the IAsys cuvette for determination of the baseline instrumental response. Protein concentrations were determined spectrophotometrically on the basis of absorption coefficients ($A^{1\%}_{1\ cm}$) of 14.4 for the lactate dehydrogenase [9], 6.6 for bovine serum albumin [10,11] and 7.35 for ovalbumin [12].

Stock solutions of sodium chloride and sucrose were prepared by direct dissolution of weighed amounts of the chemicals in distilled water.

2.3. Determination of the dead volume of the IAsys cuvette system

To assess the effectiveness of the aspiration system for liquid removal, water (100 μl) was added to the cuvette and then removed. The mass of the ''empty'' cuvette was then monitored for 16 h, and found to decrease slightly as the result of evaporation of residual water. Multiple measurements yielded a value of 10 (±1) μl for the residual volume of solvent. This dead volume, which presumably varies from instrument to instrument because of slightly different location of the aspiration device, has been taken into account in calculations of liquid-phase volume, and hence of ligate concentrations, throughout this investigation.

2.4. Free ligate contribution to biosensor response

The dependence of biosensor response upon the refractive index of the liquid phase was measured for cuvettes with unmodified carboxymethyldextran- and aminosilane-coated sensor surfaces. In a series of experiments at 21°C with sucrose and NaCl as solute, the baseline response with water (100 μl) was first measured. Successive aliquots (10 μl) of the stock solution of solute in water (14 mg/ml) were then added, and the instrumental response recorded after each addition of solute. In order to express the corresponding solute concentration in terms of a refractive index difference, $(n-n_0)$ where n_0 is the solvent refractive index, the specific refraction increments (dn/dc) of sucrose [13] and sodium chloride [14] were taken as 0.143 and 0.174 ml/g, respectively – values pertaining to a wavelength of 546 nm rather than that of 670 nm for the IAsys laser.

Similar experiments were performed with bovine serum albumin and, to a lesser extent, ovalbumin. However, for these experiments the solvent was 0.7 *M* NaCl to decrease any electrostatic interactions of the anionic proteins with either sensor surface. Specific refraction increments of 0.187 ml/g for bovine serum albumin and 0.185 ml/g for ovalbumin were calculated from values at 578 nm and the wavelength correction factor reported by Perlmann and Longsworth [15].

2.5. Response associated with saturation of the aminosilane surface with ovalbumin

Aliquots of stock ovalbumin solution (2.51 mg/ml) in dialysis equilibrium with 10 m*M* 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, pH 6.3, were added sequentially to a cuvette with an aminosilane-coated sensor surface that had been preequilibrated at 21°C with the same buffer, and the equilibrium instrumental response recorded after each addition. Values of these equilibrium responses, corrected for the contribution from free ligate concentration, were then extrapolated to infinite ovalbumin concentration to estimate the maximum response associated with saturation of the sensor surface.

2.6. Enzymatic determination of the calibration constant

An interaction between lactate dehydrogenase and the carboxymethyldextran-coated surface of an IAsys cuvette equilibrated at 21°C with 20 m*M* Tris–HCl buffer, pH 7.0, afforded a direct means of measuring the calibration constant by enzymatic determination of the extent of ligate depletion in the liquid phase. An aliquot (10 μl) of stock enzyme solution (57.6 μg/ml) was added to a carboxymethyldextran-coated cuvette containing buffer (100 μl), and the equilibrium response recorded. The bulk (80 μl) of the liquid phase was then assayed for lactate dehydro-

genase activity by monitoring the rate of NADH oxidation at 340 nm by a procedure described previously [16], except that nucleotide (rather than enzyme) addition was used to initiate reaction. This rate of NADH oxidation was then expressed relative to that for a control assay mixture with an aliquot (80 μl) of an enzyme solution with the same initial concentration (5.24 μg/ml) to determine the fraction and hence amount of enzyme bound to the sensor surface. The control solutions (100 μl) had been kept at the same temperature and in contact with the same type of surface (the top of the cuvette block) for the same length of time in an attempt to allow for any loss of ligate by adsorption to walls of the cuvette.

3. Results

Two methods have been used to determine the calibration factor relating biosensor response to the amount of a protein ligate bound to the sensor surface: (i) measurement of the response associated with saturation of the aminosilane-coated surface of an IAsys cuvette with ovalbumin under conditions of low ionic strength (10 m*M* MES, pH 6.3) to facilitate electrostatic interaction of the anionic protein with the aminosilane surface; and (ii) direct measurement by enzymatic assay of the extent of ligate depletion in the liquid phase associated with the instrumental response reflecting the interaction of lactate dehydrogenase with the carboxymethyldextran-coated surface of an IAsys cuvette (20 m*M* Tris–HCl, pH 7.0). Because the former method required the use of high protein concentrations, the contribution of free ligate concentration to biosensor response needed to be established.

3.1. Dependence of biosensor response upon free ligate concentration

Inasmuch as the biosensor response reflects the binding of ligate to matrix sites as the result of the refractive index change in the vicinity of the sensor surface, there is also a contribution from the concentration of free ligate giving rise to complex formation. Provided that the solvent for the stock ligate solution being used to supplement the contents of the IAsys cuvette is identical with that placed initially therein, the free-ligate contribution to the measured response is negligible for high-affinity ligate–matrix interactions because of the small ligate concentration (less than micromolar) required to elicit the biosensor response. On the other hand, studies of weak interactions necessitate the use of higher ligate concentrations, and hence allowance for the free-ligate contribution to the measured signal. Furthermore, this baseline shift with increasing ligate concentration can also occur in studies of high-affinity interactions in instances where precautions have not been taken to ensure identity of the solvent composition in the stock ligate solution and the reaction mixture. Supplementation of the stock solution with (say) glycerol for greater ligate stability provides a potential example of such a situation.

The biosensor response to change in the refractive index of the liquid phase has first been calibrated by examining the effects of adding aqueous solutions of sucrose and NaCl to IAsys cuvettes with carboxymethyldextran- and aminosilane-coated sensor surfaces (Fig. 1a). For either solute the biosensor response is essentially linear in refractive index change, and independent of the coating on the sensor surface. However, there is a slight discrepancy between the slopes of the dependences, 15.5 ($\pm$0.4)·10^4 and 16.3 ($\pm$0.2)·10^4 arc s, obtained with NaCl and sucrose, respectively. For bovine serum albumin and ovalbumin in 0.7 *M* NaCl (Fig. 1b) the slope of the dependence, 14.2 ($\pm$0.4)·10^4 arc s, also differs slightly from that for the other two solutes. This disparity between slopes signifies the existence of unidentified systematic error in the measurement of solute concentrations (in the absence of dry-mass determinations) and/or in the assignment of specific refractive index increments (dn/dc) at the wavelength of the laser lightsource. Nevertheless, it suffices for general purposes to combine the three sets of data into a correction factor $R_c = 15 \cdot 10^4 (n - n_0)$ arc s for the increased baseline response to a change in the bulk refractive index of the liquid phase.

From the viewpoint of the present study the more important findings are summarised in Fig. 1b, which, on the basis of an average refractive index increment of 0.185 ml/g for proteins, allows the baseline correction factor to be expressed in the form $R_c = 26(\pm 0.7)c_A$, where c_A is the mass-concentration

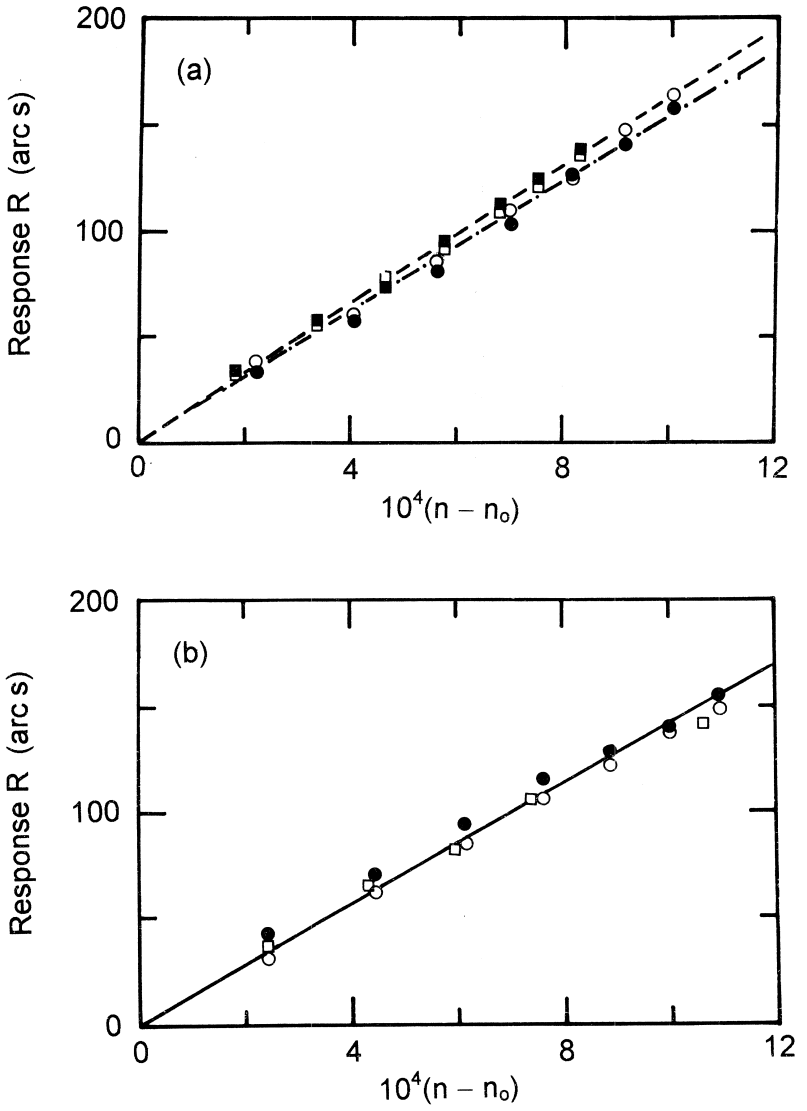

Fig. 1. Dependence of the IAsys biosensor response upon the change in refractive index of the liquid phase in cuvettes with aminosilane (open symbols) and carboxymethyldextran (closed symbols) coatings on the sensor surface. (a) Changes in the liquid-phase refractive index arising from the addition of NaCl (circles) and sucrose (squares), (b) corresponding data for bovine serum albumin (circles) and ovalbumin (squares). The lines are best-fit linear dependences of the combined data sets for sucrose (- - -), NaCl (– · –) and bovine serum albumin (—).

(mg/ml) of protein in the liquid phase. A further point of interest is that the essential identity of responses for the two sensor surfaces (Fig. 1a) still holds for a protein as large as bovine serum albumin (molecular mass 66 000). Because the polysaccharide chains extend up to 200 nm beyond the sensor surface of the carboxymethyldextran-coated cuvette [17], any appreciable gel-partitioning effect [18] would lead to a decreased free-ligate concentration in the immediate vicinity of the sensor surface, and hence to a lower bulk refractive index response than that observed with the aminosilane surface. The lack of distinction between the bulk refractive index responses for the two cuvettes (Fig. 1b) thus indicates a value close to unity for the gel-partition coefficient of serum albumin. Inasmuch

as the equilibrium constant measured by biosensor technology is the product of the binding constant and the partition coefficient [19], its consideration as a measure of the affinity is seemingly validated for moderately sized macromolecular ligates.

3.2. Calibration of IAsys response in terms of the amount of bound protein ligate

The initial attempt to determine the calibration constant relating IAsys response to the amount of bound protein ligate entailed estimation of the maximum response attained by saturation of an aminosilane-coated sensor surface. From the concentration dependence of binding response (corrected for the free-ligate contribution) obtained for ovalbumin in 10 m*M* MES buffer (pH 6.3), this maximum response is in the vicinity of 200 arc s (Fig. 2), a more definitive estimate being precluded by the unavailability of an analytical relationship to guide the extrapolation of biosensor response to infinite concentration of free ligate ($1/c_A \rightarrow 0$). However, a value of 200 (± 5) arc s is consistent with either extrapolation procedure attempted in Fig. 2. This maximum response now needs to be combined with an estimate of the amount of ovalbumin that is commensurate with saturation of the sensor surface.

X-Ray crystallography has shown ovalbumin to be a triaxial ellipsoid with semi-axes of 3.5, 2.5 and 2.25 nm [20]. However, we simplify the calculations by approximating the protein as a prolate ellipsoid of revolution with semi-minor axes of 2.37 nm; and by assuming that its anionic charge is spread uniformly over the ellipsoid surface. Because there is then no preferred orientation for the protein's general electrostatic interaction with the positively charged aminosilane surface, entropic considerations dictate that the ovalbumin molecules be aligned with their semi-

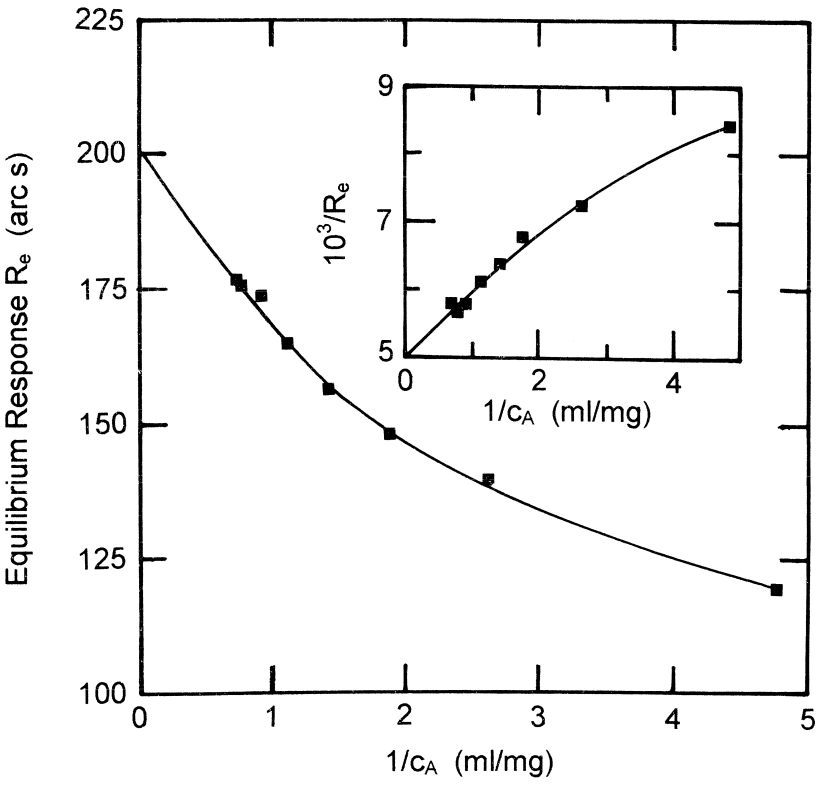

Fig. 2. Estimation of the maximum biosensor response associated with saturation of an aminosilane-coated sensor surface with ovalbumin (10 m*M* MES buffer, pH 6.3) by extrapolation to infinite ligate concentration ($1/c_A \rightarrow 0$). Inset: corresponding extrapolation in double-reciprocal format.

major axes perpendicular to the surface in order to attain surface saturation. During the course of surface saturation molecules will inevitably bind to the surface in all orientations; and hence it might be argued that saturation might represent a lower packing density than is being postulated here. However, such suboptimal packing merely represents a transient state en route to thermodynamic equilibrium. Consequently, at equilibrium ovalbumin should pack in the same manner as a circular disk with $r=2.37$ nm. Calculations based on an accessible area that is 90.7% of the total surface area for a circular ligate [21,22] yield an estimate of $5.1\cdot10^{10}$ molecules/mm^2 (3.8 ng/mm^2 for a protein with a molecular mass of 45 000) for the amount of bound ovalbumin at surface saturation. Taken in conjunction with the estimated saturation response of 200 arc s (Fig. 2), a calibration factor of 52 arc s ng^{-1} mm^2 for proteins is indicated.

As an independent check on this interpretation of the ovalbumin data, a second estimate of the calibration constant has been obtained by direct measurement of the extent of ligate depletion as the result of interaction between lactate dehydrogenase and the unmodified carboxymethyldextran-coated surface of an IAsys cuvette (20 m*M* Tris–HCl, pH 7.0). Triplicate experiments with 576 ng enzyme yielded an estimate of 0.58 ($\pm$0.09) for the fraction of lactate dehydrogenase remaining in the liquid phase after attainment of equilibrium. Combination of this finding with the binding responses of 687 ($\pm$26) arc s for a sensor surface with an area of 20 mm^2 [2] leads to a value of 57 ($\pm$15) arc s ng^{-1} mm^2 for the calibration constant. In view of the close agreement with the above estimate, we conclude that a calibration constant of 50–60 arc s ng^{-1} mm^2 is a more appropriate proportionality factor than the earlier values of 163 arc s ng^{-1} mm^2 [2] and 206 arc s ng^{-1} mm^2 [3] for the interpretation of the IAsys response in terms of amount of bound protein ligate.

4. Discussion

This investigation has served several useful roles. (i) On a purely technical note it has highlighted the need to check whether the IAsys aspiration system removes all of the liquid from the cuvette: for the present instrument the residual volume after liquid removal by aspiration amounted to 10 μl. (ii) This study has drawn attention to the importance of allowing for the contribution to biosensor response that emanates from changes in the bulk refractive index of the liquid phase in instances where high ligate concentrations are required to offset a low-affinity matrix–ligate interaction. (iii) The finding that this free-ligate contribution to instrument response is the same for bovine serum albumin in IAsys cuvettes with carboxymethyldextran- and aminosilane-coated sensor surfaces supports the contention [23] that the array of polysaccharide chains is sufficiently flexible to render negligible any difference between free ligate concentrations in the liquid and gel phases in biosensor studies with carboxymethyldextran-coated sensor surfaces. (iv) Finally, studies of the relationship between instrument response and the amount of bound protein ligate have led to a revised estimate of 50–60 (cf. 163 and 206) arc s ng^{-1} mm^2 for the calibration constant.

An important consequence of this last finding is that the extent of ligate depletion in IAsys studies has been about three- to four-fold greater than was thought previously [5–8]. Recent conclusions about the negligibility of the effect for many systems [8] need reconsideration. As noted previously [7,8], neglect of the effect of ligate depletion gives rise to underestimation of the binding constant (determined by thermodynamic or kinetic means) because of the overestimate of the free ligate concentration that is incorporated into its determination. In that regard the development of a double-cuvette system has lessened the extent of the depletion problem by virtue of a four- to five-fold decrease in sensor-surface area (4 mm^2 cf. 16 or 20 mm^2): the extent of ligate depletion is thus likely to be similar to that deduced for the older cuvette system on the basis of the original calibration constant. In any event, it now appears that allowance for the effects of ligate depletion in the liquid phase should be incorporated routinely into kinetic and thermodynamic studies of interactions by IAsys biosensor technology – a task that can be accomplished by a version of the computer software being developed by the manufacturer.

Acknowledgements

The support of this investigation by the Australian Research Council is gratefully acknowledged, as is the receipt (by D.R.H.) of a University of Queensland Postgraduate Award.

References

[1] R. Cush, J.M. Cronin, W.J. Stewart, C.H. Maule, J. Molloy, N.J. Goddard, Biosens. Bioelectron. 8 (1993) 347.
[2] R.J. Davies, P.R. Edwards, H.J. Watts, C.R. Lowe, P.E. Buckle, D. Yeung, T.M. Kinning, D.V. Pollard-Knight, in: J.W. Crabb (Ed.), Techniques in Protein Chemistry V, Academic Press, San Diego, CA, 1995, p. 285.
[3] P.R. Edwards, A. Gill, D.V. Pollard-Knight, M. Hoare, P.A. Lowe, R.J. Leatherbarrow, Anal. Biochem. 231 (1995) 210.
[4] P.R. Edwards, P.A. Lowe, R.J. Leatherbarrow, J. Mol. Recogn. 10 (1997) 128.
[5] D.R. Hall, D.J. Winzor, Anal. Biochem. 244 (1997) 152.
[6] D.R. Hall, N.N. Gorgoni, J.G. Altin, D.J. Winzor, Anal. Biochem. 253 (1997) 145.
[7] D.R. Hall, D.J. Winzor, J. Chromatogr. B 715 (1998) 163.
[8] P.R. Edwards, C.H. Maule, R.J. Leatherbarrow, D.J. Winzor, Anal. Biochem. 263 (1998) 1.
[9] R. Jaenicke, S. Knof, Eur. J. Biochem. 4 (1968) 157.
[10] E.J. Cohn, W.L. Hughes Jr., J.H. Weare, J. Am. Chem. Soc. 69 (1947) 1753.
[11] C. Tanford, G.L. Roberts Jr., J. Am. Chem. Soc. 74 (1952) 2509.
[12] L.W. Cunninham Jr., B.J. Nuenke, J. Biol. Chem. 234 (1959) 1447.
[13] L.J. Gosting, M.S. Morris, J. Am. Chem. Soc. 71 (1949) 1998.
[14] V. Vitagliano, P.A. Lyons, J. Am. Chem. Soc. 78 (1956) 1549.
[15] G.E. Perlmann, L.G. Longsworth, J. Am. Chem. Soc. 70 (1948) 2719.
[16] D.R. Hall, M.P. Jacobsen, D.J. Winzor, Biophys. Chem. 57 (1995) 47.
[17] B. Johnsson, S. Löfäs, G. Lindquist, Å. Edström, R.-M. Müller Hillgren, A. Jansson, J. Mol. Recogn. 8 (1995) 125.
[18] P. Schuck, Biophys. J. 70 (1996) 1230.
[19] D.J. Winzor, J. Chromatogr. A 803 (1998) 291.
[20] P.E. Stein, A.G. Leslie, J.T. Finch, R.W. Carrell, J. Mol. Biol. 221 (1991) 941.
[21] F.C. Andrews, J. Chem. Phys. 64 (1976) 1941.
[22] S. Stankowski, Biochim. Biophys. Acta 735 (1983) 352.
[23] R. Karlsson, A. Fält, J. Immunol. Methods 200 (1997) 121.