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# NATIVE AND HYDROPHOBIZED HUMAN IgG
## Enthalpies of heat-induced structural changes and adsorption onto silica

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## Abstract

Differential scanning calorimetry (DSC) and isothermal calorimetric batch technique were used to monitor the heat-induced structural changes and adsorption properties of human immunoglobulin G (IgG), in native and hydrophobized states. The transition temperature ($T_{max}$) and enthalpy of heat-induced conformational changes ($\Delta_{cal}H$) of IgG in solution as well as the enthalpy change accompanying the adsorption of IgG onto hydrophilic silica ($\Delta_{ads}H$), were shown to depend on the degree of the protein hydrophobicity (number of covalently attached alkyl chains). The adsorption enthalpy for all forms of IgG at all surface concentrations was found to be endothermic, that is the process is entropy driven. Factors affecting the IgG adsorption onto silica are discussed.

**Keywords:** enthalpy of adsorption, human IgG, structural changes

## Introduction

The ability to control conformational stability of globular proteins at different physicochemical conditions, and to control their adsorption onto solids surface are of great importance in various applications such as enzyme immobilization and chromatography [1–7], emulsification and foaming [8, 9].

Immunoglobulins are used as analytical reagents with very high specificity and binding affinity. Immunoglobulin G (IgG) molecules are composed of four polypeptide chains: two 'heavy' with 446 amino acid residues (~50 kDa) and two 'light' with 214 amino acid residues (~25 kDa). These chains are cross-linked by disulfide bonds into the 'T'- or 'Y'-shaped structure, which is characterized by three fragments (two $F_{ab}$ and one $F_c$). The secondary structure of the IgG molecule is characterized by different structural elements, at least twelve domains, composed mainly of antiparallel β-sheets and β-turns stabilized by hydrogen bonding [10–13].

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It has been found that chemical hydrophobization of IgG by attachment of alkyl chains leads to appreciable changes in its surface activity and functional properties. Hydrophobized human IgG spontaneously aggregates in aqueous solutions, and the size of aggregates (colloidal clusters) depends both on the number of attached alkyl chains and on their length. It displays higher affinity for hydrophobic surfaces of polystyrene and silica coated by phosphatidyl choline monolayer and forms more compact surface layers as compared with the native protein [14, 15]. Modified IgG decreases the surface tension at the air/water interface more effectively than the native protein, and this decrease correlates with an increase in the surface hydrophobicity index (SHI) evaluated with the use of fluorescent hydrophobic probe, 8-anilino-1-naphthalenesulfonate [16]. In addition, hydrophobized human IgG retains high specific recognition ability in ELISA tests [14].

An important question is also how the increase in hydrophobicity by the chemical modification affects the structural characteristics and conformational stability of proteins.

There are several experimental approaches [17–20] providing information on the conformational changes in the protein molecules (optical rotation dispersion, far-UV circular dichroism, Raman spectroscopy, and DSC). This last technique allows the determination of the thermodynamic parameters of conformational transitions directly from the heat capacity curves, providing an information on the thermodynamic features of protein unfolding and on the contribution of different forces determining a protein stability. The heat-induced structural changes in IgG molecules were the focus of few studies [17, 20–22].

The adsorption behavior of proteins at various solids is a result of multiple interactions between the components of the system, such as adsorbent surface, the protein molecule, the solvent and the other solutes, which are present in the solution. Calorimetry allows direct measurement of the adsorption enthalpy, $\Delta_{ads}H$, and evaluation of the entropy change, $\Delta_{ads}S$. Based on this data, one can draw conclusions on possible mechanisms of the protein adsorption [2, 23–25]. Elucidation of the physicochemical parameters governing the adsorption of the native and modified IgG is of great importance for improving the existing and creating the new immunodiagnostic kits, since adsorption of immunoglobulins onto solids is a necessary step in various types of immunoassays.

In our recent publications we used DSC and isothermal batch microcalorimetry to evaluate two aspects: a) The correlation between heat-induced structural changes and changes in hydrophobicity for the native and chemically modified molecules of human IgG [26], and b) The enthalpy of adsorption of IgG with various degrees of hydrophobicity onto silica [27].

The aim of the present paper is to present how microcalorimetry can be used in studying the effect of chemical hydrophobization of the IgG molecules on their conformational stability in solution, and during adsorption onto hydrophilic surface.

## Experimental section

*IgG isolation and modification*

IgG was isolated from human serum by ammonium sulfate precipitation, purified by caprylic acid, and concentrated by additional ammonium sulfate precipitation [28]. The IgG modification was performed by covalent attachment of lauroyl residues to the protein lysine amino groups by reaction with N-lauroyloxysuccinimide [14]. The ester was synthesized and isolated according Lapidot *et al.* [29].

The number of covalently bound alkyl chains after modification was determined by the TNBS method [30, 31]. The samples used for differential scanning calorimetry experiments were characterized by an average of 4, 12 and 19 attached alkyl chains per protein molecule ($4C_8$-IgG, $12C_8$-IgG and $19C_8$-IgG, respectively), while in adsorption experiments the samples containing 11, 25 and 52 alkyl chains per protein molecule ($11C_8$-IgG, $25C_8$-IgG and $52C_8$-IgG, respectively) were used.

Protein concentrations were determined spectrophotometrically (Hitachi double-beam spectrophotometer, Model U-2000) by the Bradford method [32] or by the improved Lowry method [33] using Bio-Rad protein assay reagents.

*Differential scanning calorimetry*

Micro-DSC III (Setaram, Caluire-France) was used to monitor the heat induced structural changes in the native and chemically modified IgG. The protein solution (2.8 mg $mL^{-1}$) in 0.1 M PBS (phosphate buffer saline), pH 7.4, was used for measurements. The experimental details are described in [26].

The heat conformational stability of the native and chemically modified IgGs was evaluated from the values of the initial ($T_i$) and maximum ($T_{max}$) deviations of the heat flow signal and also from the value of specific enthalpy change ($\Delta_{cal}H$), which was calculated from the area under the peak transition (after subtraction of the sample baseline) with the use of a straight baseline between the initial and final temperatures of the peak transition [19].

*Adsorption of IgG*

IgG adsorption on silica was performed from 0.1 M PBS (phosphate saline buffer), pH 7.4 at 25°C as described in [27]. The adsorption isotherms were assessed by a depletion method by measuring the bulk protein concentrations before and after adsorption.

*Isothermal calorimetry*

The enthalpy changes accompanying the adsorption of immunoglobulins onto silica were measured by an isothermal microcalorimetric batch technique that allowed the adsorbing species to be introduced from the outside of the calorimeter into the calorimetric cell containing a homogeneous suspension of solid in the solvent [34]. The differential enthalpies associated with the subsequent adsorption steps and thereby corresponding to any change

in the adsorbed phase during its formation were calculated from the experimentally measured enthalpy changes, taking the effect of dilution into account as described in [27].

In order to cover the whole adsorption range, i.e. from the beginning of the isotherm to its plateau saturation, concentrated stock solutions of the protein at our experimental conditions should be used (construction of the calorimetric cell used in these experiments demands large volume of the protein solution). However, we were limited by the solubility of the modified immunoglobulins in the buffer solution (the maximum protein concentrations used were about 5.5–6.0 mg $mL^{-1}$), therefore the adsorption enthalpies were measured up to surface coverage ($\Gamma/\Gamma_{max}$) of 0.6.

*Immunological activity*

The immunological activity of IgG was tested with the use of conventional ELISA procedure [35]. The detailed description of immunotests is given in [27].

## Results and discussion

*Heat-induced transitions*

The first heating DSC curve (0.1°C $min^{-1}$) for native IgG after subtraction of the buffer curve, shows a single endothermic transition spreading between 60 and 80°C with the peak at 68°C and the specific transition enthalpy 21 J $g^{-1}$ (Fig. 1a). This transition corresponds to thermal protein unfolding. The nonsymmetrical shape of the transition peak could be due to a multistep behavior, as expected for such multidomain protein as IgG.

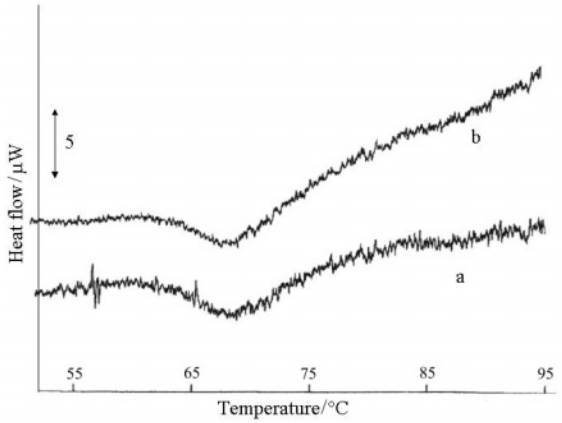


**Fig. 1** DSC curve for the native IgG (2.8 mg $mL^{-1}$ of the protein in 0.1 M PBS, pH 7.4; heating from 10 to 100°C at 0.1°C $min^{-1}$)

The curve for IgG with 4 and 12 attached caprylic chains were also characterized by endothermic transitions with peaks located at 68°C for $4C_8$-IgG but at a lower tem-

perature (66°C) for $12C_8$-IgG. The corresponding $\Delta_{cal}H$ values decreased with an increase in the number of attached caprylic chains (20 and 17 J $g^{-1}$ for $4C_8$-IgG and $12C_8$-IgG, respectively). The transition peak for IgG with 19 attached alkyl chains was not repeatable, and we consider its $\Delta_{cal}H$ value equal to zero. The overall heat of reaction is due to positive contribution of the hydration of hydrophobic groups and to negative contribution of the hydration of polar groups as well as subsequent protein-protein interactions, which can take place in the same transition temperature range [17–19, 36]. In our previous study [26] we observed for 1°C $min^{-1}$ heating rate a drastic change in DSC curve, with a large distortion due to an exothermic peak corresponding to aggregation of unfolded protein molecules with maximum at 73°C. The absence of aggregation peak at low heating rate was explained by assuming that in this case at any time only a low concentration of denatured protein molecules is available for aggregation, while at higher heating rates a much higher concentration of denatured molecules is available, resulting in rapid formation of very compact aggregates [17]. For hydrophobized IgGs the temperatures corresponding to the initial and maximal deviations of the heat flow as well as the area under the transition peak decrease as the number of attached alkyl chains increases. As seen from Table 1, the decrease in relative values of the enthalpy of heat-induced conformational transitions for hydrophobically modified and native IgGs correlates with an increase in the relative values of the surface hydrophobicity index, as determined previously [26, 37]. Thus, the decrease in the heat of IgG unfolding is accompanied by enhanced exposure of initially buried groups of the protein molecules to the aqueous medium. These findings may be explained by weakening of the intramolecular interactions responsible for the rigidity of the IgG molecules with simultaneous strengthening of the protein-protein interactions as previously reported for other globular proteins under the effect of heat denaturation [37].

**Table 1** Variations of enthalpy of heat-induced conformational transition ($\Delta_{cal}H$) and surface hydrophobicity index (SHI) for hydrophobically modified human IgG, relative to the native IgG (calculations are based on data from [26]. Upper indices M and N correspond to the modified and native IgGs, respectively

| IgG | $\Delta_{cal}H^M/\Delta_{cal}H^N$ | $SHI^M/SHI^N$ |
|---|---|---|
| Native IgG | 1.00 | 1.00 |
| $4C_8$-IgG | 0.95 | 1.85 |
| $12C_8$-IgG | 0.80 | 5.40 |
| $19C_8$-IgG | – | 6.40 |

*Calorimetric characterization of IgG adsorption*

The adsorption isotherms presented in Fig. 2 indicate a high affinity of all forms of the protein for the silica. The plateau surface concentration increases in the order native IgG (~$1.65\cdot10^{-8}$ mole $m^{-2}$)<$11C_8$-IgG (~$1.95\cdot10^{-8}$ mole $m^{-2}$)<$25C_8$-IgG (~$2.2\cdot10^{-8}$ mole $m^{-2}$) and then decreases for IgG with 52 attached alkyl chains (~$1.4\cdot10^{-8}$ mole $m^{-2}$). These val-

ues correspond to the molecular cross section areas of 10000, 8500, 7500 and 12000 Å$^2$ for the native IgG, 11$C_8$-IgG, 25$C_8$-IgG and 52$C_8$-IgG, respectively. According to X-ray diffraction data, the dimensions of 'cap' and 'leg' of T-shaped IgG molecule are 142×50×40 and 45×45×38 Å, respectively [11]. Therefore, the molecular area of the native T-shaped IgG in a compact monolayer should be equal to 7100 Å$^2$ in the case of 'cap'-on and 'leg'-on disposition of the protein molecule on the surface, that corresponds to surface concentration of ~$2.3 \cdot 10^{-8}$ mole m$^{-2}$ which is comparable with experimentally found values.

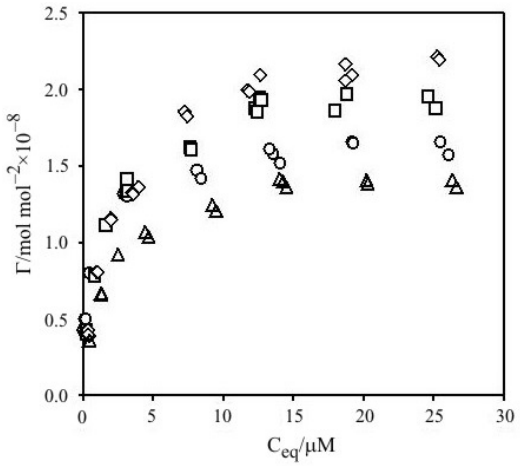


**Fig. 2** Adsorption isotherms for o – native IgG, □ – 11$C_8$-IgG, ◇ – 25$C_8$-IgG and △ – 52$C_8$-IgG on silica (0.1 M PBS, pH 7.4, 25°C)

Adsorption of proteins at solid/liquid interfaces is controlled by various contributions: electrostatic interactions between the surface and the protein as well as between the protein molecules in the surface layer, van der Waals forces, hydrophobic interactions, structural rearrangements and conformational changes, changes in the state of hydration of the sorbent and protein surfaces, rearrangement of the excluded water or ion molecules in a bulk solution and redistribution of charged groups in the interfacial layer [2, 23–25, 38]. Regardless of the mechanism, adsorption of proteins takes place only if the Gibbs energy of the system decreases ($\Delta_{ads}G=\Delta_{ads}H-T\Delta_{ads}S<0$), were $G$, $H$, $S$ and $T$ are the Gibbs energy, enthalpy, entropy and absolute temperature, respectively. Calorimetry allows measurement of $\Delta_{ads}H$ directly. The sign and magnitude of the adsorption enthalpy are governed by a competition between above-mentioned subprocesses, and $\Delta_{ads}H$ shows, as a rule, complex dependencies on the surface concentration, pH and temperature.

Figure 3 shows the isothermal enthalpy for the native and hydrophobically modified human IgG adsorption on silica as a function of surface coverage ($\Gamma/\Gamma_{max}$). For all forms of IgG at all surface concentrations studied, the adsorption is endothermic, that is the process is entropy driven. These results are in agreement with previously published data on adsorption of a number of proteins on various sorbents, such as

RNase on negatively charged polystyrene [23], myoglobin and HPA on hematite [39, 40], bovine serum albumin on hydrophobized Sepharose at high surface concentrations [5], on silica-based cross-linked polyethyleneimine cation-exchanger resin [6], and on cellulosic quaternary amine-bearing anion-exchanger [41] and recombinant soluble tryptic fragment of rat cytochrome $b_5$ on quaternary amine-based anion-exchanger [42].

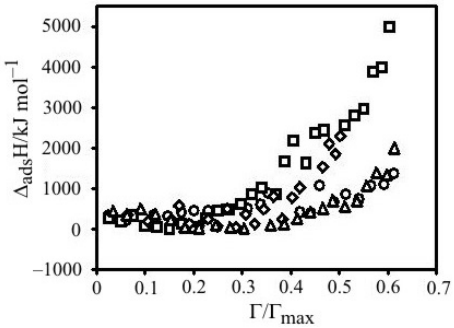


**Fig. 3** Enthalpy of adsorption of o – native IgG, □ – $11C_8$-IgG, ◇ – $25C_8$-IgG and △ – $52C_8$-IgG as a function of the surface coverage

The positive $\Delta_{ads}H$ values for various forms of IgG are nearly constant and low at low surface coverage (up to about 25–30%). At higher surface concentrations, however, significant endothermic heats are observed. Adsorption of $11C_8$-IgG is the most endothermic, followed by adsorption of $25C_8$-IgG, while the adsorption enthalpies for the native IgG and $52C_8$-IgG are the least endothermic and very similar at all surface coverages.

The main contributions to the positive values of $\Delta_{ads}S$ may arise from dehydration of the sorbent and protein surfaces and from structural changes in the protein molecules upon adsorption. As silica has hydrophilic surface, dehydration of the sorbent will not occur to a significant extent, but dehydration of exposed hydrophobic sites of the IgG molecules might sufficiently contribute to the adsorption entropy. Attachment of alkyl chains to IgG molecules should result in an increase in contribution of the positive dehydration term to entropy and in simultaneous increase in the positive enthalpy of the process at high surface coverage due to electrostatic repulsion of identically charged protein molecules, since the net negative charge of the modified protein molecules is greater than that of the molecules of native IgG ($\zeta$ potentials of the native, $11C_8$-, $25C_8$-, and $52C_8$-IgGs in 0.1 PBS, pH 7.4, are –4.5, –6.4, –9.9 and –16.2 mV, respectively). Dehydration may be followed by hydrophobic interactions between alkyl chains of adjacent protein molecules that results in formation of more compact surface layer.

The large entropy increase driving the unfolding of globular proteins in aqueous solutions can also drive the protein adsorption. The enthalpy change in the protein unfolding process is, usually, large and endothermic due to the loss of favorable intramolecular interactions.

Micro-DSC curves of proteins adsorbed on hydrophilic sorbents, including silica, indicate changes in the protein structure upon adsorption [38, 43–45].

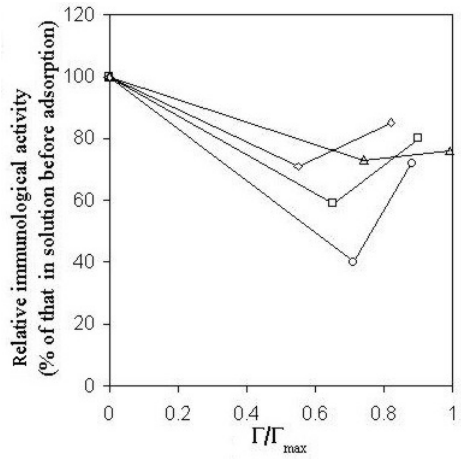


**Fig. 4** Immunological activity of the native IgG (o), $11C_8$-IgG (□), $25C_8$-IgG (◇) and $52C_8$-IgG (△) desorbed from silica as a function of the surface coverage.

Irreversible structural changes in adsorbed molecules were confirmed by reduction of immunological activity of all forms of IgG desorbed from silica as compared with the corresponding forms before adsorption (Fig. 4). This reduction was more pronounced at low surface concentrations, when there is enough room for unfolding of the protein molecules on the surface, while at a high surface concentrations the protein molecules are sterically hindered from unfolding.

Thus, from the results presented in this manuscript, it may be concluded that hydrophobization of the protein molecules by covalent attachment of alkyl chains results in noticeable changes in conformational stability of proteins in solution. The changes in conformational stability also influence the adsorption of proteins at hydrophilic negatively charged silica surfaces in terms of the adsorbed amount and calorimetric effects.

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## References

1 A. Kamyshny, Russ. J. Phys. Chem., 55 (1981) 319.
2 C. A. Haynes and W. Norde, Colloids Surf., B 2 (1994) 517.
3 J. L. Brash and T. A. Horbett, In Proteins at Interfaces II. Fundamentals and Applications. T. A. Horbett and J. L. Brash Eds ACS Symposium Series, American Chemical Society, Washington DC, 602 (1994) 1.
4 S. Magdassi and A. Kamyshny, In Surface Activity of Proteins: Chemical and Physicochemical Modifications. S. Magdassi Ed. Marcel Dekker, New York, 1996, Chapter 1, p. 1.

5 M. A. Esquibel-King, A. C. Dias-Cabral, J. A. Queiroz and N. G. Pinto, J. Chromatogr., 865 (1999) 111.
6 P. Raje and N. G. Pinto, J. Chromatogr., 796 (1988) 141.
7 F.-Y. Lin, W.-Y. Chen, R.-C. Ruaan and H.-M. Huang, J. Chromatogr., 872 (2000) 37.
8 E. Dickinson, Introduction to Food Colloids, Oxford University Press, Oxford 1992.
9 S. Magdassi and O. Toledano, In Surface Activity of Proteins: Chemical and Physicochemical Modifications. S. Magdassi Ed. Marcel Dekker, New York 1996, Chapter 2, p. 39.
10 V. R. Sarma, E. W. Silverton, D. R. Davis and W. D. Terry, J. Biol. Chem., 246 (1971) 3753.
11 I. Pilz, O. Kratky and F. Karush, Eur. J. Biochem., 41 (1974) 91.
12 J. Deisenhofer, Biochemistry, 20 (1981) 2361.
13 M. Marquart, J. Deisenhofer, R. Huber and W. Palm, J. Mol. Biol., 141 (1980) 369.
14 A. Kamyshny and S. Magdassi, Colloids Surf., B 9 (1997) 147.
15 A. Kamyshny, O. Toledano and S. Magdassi, Colloids Surf., B 13 (1999) 187.
16 A. Kamyshny, S. Magdassi and P. Relkin, J. Colloid Interface Sci., 212 (1999) 74.
17 A. W. P. Vermeer, G. E. G. Bremer and W. Norde, Biochim. Biophys. Acta, 1425 (1998) 1.
18 P. L. Privalov, Adv. Protein Chem., 33 (1979) 167.
19 J. Lefebvre and P. Relkin, In Surface Activity of Proteins: Chemical and Physicochemical Modifications. S. Magdassi Ed. Marcel Dekker, New York 1996, Chapter 7, p. 181.
20 V. M. Tishchenko, V. P. Zav'yalov, G. A. Medgyesi, S. A. Potekhin and P. L. Privalov, Eur. J. Biochem., 126 (1982) 517.
21 J. Buchner, M. Renner, H. Lilie, H.-J. Hinz, R. Jaenicke, T. Kiefhaber and R. Rudolf, Biochemistry, 30 (1991) 6922.
22 S. P. Maltsev, Z. I. Kravchuk, A. P. Vlasov and G. Lyakhnovich, FEBS Lett., 361 (1995) 173.
23 W. Norde and J. Lyklema, J. Colloid Interface Sci., 66 (1978) 295.
24 W. Norde, J. Disp. Sci. Technol., 13 (1992) 363.
25 F. Galisteo and W. Norde, Colloids Surf., B 4 (1995) 375.
26 P. Relkin, A. Kamyshny and S. Magdassi, J. Phys. Chem., B 104 (2000) 4980.
27 A. Kamyshny, S. Lagerge, S. Partyka, P. Relkin and S. Magdassi, Langmuir, 17 (2001) 8242.
28 E. Harlow and D. Lane, Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, New York 1988, p. 283.
29 Y. Lapidot, S. Rappaport and Y. Wolman, J. Lipid Res., 8 (1967) 142.
30 A. F. S. Habeeb, Anal. Biochem., 14 (1966) 328.
31 J. Adler-Nissen, Agric. Food Chem., 27 (1979) 1256.
32 J. Bradford, Agric. Food Chem., 27 (1979) 248.
33 G. L. Peterson, Anal. Biochem., 100 (1979) 201.
34 S. Partyka, M. Lindheimer, S. Zaini, E. Keh and B. Braun, Langmuir, 2 (1986) 101.
35 E. Harlow and D. Lane, Antibodies. A Laboratory Manual. Cold Spring Harbor Laboratory, New York, 1988, p. 553.
36 A. K. P. Murphy and E. Freire, Adv. Protein Chem., 222 (1991) 687.
37 P. Relkin, Int. J. Biol. Macromol., 22 (1997) 59.
38 W. J. Norde and J. P. Favier, Colloids Surf., 64 (1992) 87.
39 T. Arai and W. Norde, Colloids Surf., 51 (1990) 1.
40 P. G. Koutsoukos, W. Norde and J. Lyklema, J. Colloid Interface Sci., 95 (1983) 385.
41 W. R. Bowen and D. T. Hughes, J. Colloid Interface Sci., 158 (1993) 395.
42 D. S. Gill, D. J. Roush, K. A. Shick and R. C. Willson, J. Chromatogr., 715 (1995) 81.
43 C. A. Haynes and W. Norde, J. Colloid Interface Sci., 169 (1995) 313.

44 B. L. Steadman, K. C. Thomson, C. R. Middaugh, K. Matsuno, S. Vrona, E. Q. Lawson and R. V. Lewis, Biotechnol. Bioeng., 40 (1992) 8.
45 H. Larsericsdotter, S. Oscarsson and J. J. Buijs, J. Colloid Interface Sci., 237 (2001) 98.