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Capillary electrophoresis-based analysis of phospholipid and glycosaminoglycan binding by human β_2-glycoprotein I☆

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Abstract

Human β_2-glycoprotein I ($\beta_2$gpI) is a phospholipid and heparin binding plasma glycoprotein involved in autoimmune diseases characterized by blood clotting disturbances (thrombosis) together with the occurrence of autoantibodies against $\beta_2$gpI. With the final goal of assessing autoantibody influence on binding interactions of $\beta_2$gpI we have studied the development of capillary electrophoresis (CE)-based assays for interactions of negatively charged ligands with $\beta_2$gpI. In the development of suitable conditions for analysis at neutral pH of this basic protein (p*I* about 8) we found the pH hysteresis behavior of fused silica surfaces useful since the protonated surface after an acid pre-wash counteracted protein adsorption efficiently in contrast to more laborious procedures including acrylamide/dimethylacrylamide coatings that did not permit analysis of this particular protein. This simple approach made estimates of heparin–$\beta_2$gpI interactions possible and the principle was shown also to work for detection of $\beta_2$gpI binding to anionic phospholipids. Utilizing the pH hysteresis effect may be a simple solution to the adsorption problems often encountered in analyses of proteins by CE.
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1. Introduction

The characterization of interactions between biological molecules may be accomplished by capillary electrophoresis under non-denaturing conditions [1]. Binding that leads to changes in analyte peak areas and migration times gives both qualitative and quantitative information on molecular interactions and this has emerged as a useful approach especially when analytical material is scarce, when resolution is pivotal, and when the interaction kinetics are suitable [1–3]. CE-based binding studies have been biased towards small molecule interactions such as those involving peptides, other small biomolecules, and drugs [1]. One important reason for this is probably that protein analytes often exhibit recovery problems because of interactions with the inner surface of fused silica capillaries. This surface is highly negatively charged at the neutral pH that is required for non-denaturing analyses. This leads to absent, irreproducible, or tailing peaks that preclude reliable estimates of, e.g. binding constants. The detrimental wall-charge originates from the ionized silanol groups of the quartz glass [4]. Numerous charge suppression strategies have been proposed to enable general protein analysis [5,6] including covalent coatings of acrylamide and acrylamide derivatives [7,8]. Other approaches require extremes of pH or ionic strength but are discounted for physiologically relevant binding studies.

In this study we work out conditions conducive to CE-based analyses of the human plasma protein β_2-glycoprotein I ($\beta_2$gpI or apolipoprotein H) that has a p*I* of approximately

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8.0 [9] and accordingly is not recovered by CE in uncoated fused silica capillaries in plain neutral electrophoresis buffers. $\beta_2$gpI has specific anionic phospholipid and glycosaminoglycan binding capabilities and may be involved in the clearance of apoptotic cells but its precise physiological role has not been established [10]. The binding interactions of $\beta_2$gpI are of interest also because this protein participates in coagulation processes and is involved in autoimmune thrombophilic diseases, i.e. conditions, so-called anti-phospholipid syndromes, where a propensity to form blood clots is associated with circulating autoantbodies against $\beta_2$gpI [11–18]. The protein is a M_r 55 000 fish hook-shaped single chain, five domain glycoprotein [19]. Most autoantibodies appear to be binding at sites at the N-terminus of the protein while phospholipid binding is effected by domain V in the C-terminal part [20–24]. Almost nothing is known about if and how anti-$\beta_2$gpI autoantibodies may cause dysfunction of this protein, e.g. by modulating its binding interactions with natural ligands. As a foundation for subsequent studies of the structure and function of $\beta_2$gpI and of pathogenetic mechanisms in anti-phospholipid syndromes it is therefore of interest to establish conditions for reproducible analysis of $\beta_2$gpI and its interactions with anionic phospholipids and glycosaminoglycans. We here show how reproducible recovery of $\beta_2$gpI in CE analyses may be obtained exploiting the pH hysteresis effect where the charge on the capillary surface is controlled by the preconditioning pH [25,26]. Further, we show how, under the conditions we have worked out, affinity electrophoresis experiments with $\beta_2$gpI and its ligands heparin and anionic phospholipids can be performed. While the approach proved very efficient in our specific application its use as a more generally applicable method for protein analysis by CE will require more thorough characterization.

2. Experimental

2.1. Materials

Bovine lung heparin (BLH) (average molecular mass: 15 000) was obtained from Calbiochem (Darmstadt, Germany); aprotinin was obtained from Roche (Basel, Switzerland); L-α-phosphatidyl-L-serine (PS), γ-methacryloxypropyltrimethoxysilane (MPT), acrylamide, *N*,*N*-dimethylacrylamide (DMA), phosphoric acid, ammonium persulfate (APS) and *N*,*N*,*N*′,*N*′-trimethylethylenediamine (TEMED) were obtained from Sigma–Aldrich (St. Louis, MO, USA); 1-palmiotyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) from Larodan (Malmö, Sweden). All other chemicals were of analytical grade. The liposome solution was prepared as described in [27–30].

2.2. Purification of human $\beta_2$gpI

$\beta_2$gpI was purified from outdated human plasma by perchloric acid precipitation [31] followed by heparin-Sepharose affinity chromatography, anion exchange chromatography, and size-exclusion chromatography. Purity was ascertained using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and crossed immunoelectrophoresis as described [11]. Protein concentration was determined by the Lowry assay using bovine serum albumin as a standard.

2.3. Capillary electrophoresis

Fused silica capillaries with a 50 μm internal diameter were obtained from Polymicro Technologies (Phoenix, AZ, USA) or from Beckman (Fullerton, CA, USA). Acrylamide-coated capillaries were prepared according to Hjertén with some modifications [8]. The capillaries were filled with 0.5 M NaOH and kept for 3 h at room temperature, followed by rinsing with water, 0.1 M HCl and water again. Then the capillaries were filled with 3% (v/v) MPT-solution in 60% (v/v) acetone and kept overnight. This solution was replaced with 3% (w/v) acrylamide solution containing 0.05% (v/v) TEMED and 0.05% (w/v) APS and the capillaries were again kept overnight. The capillaries were then filled with water and stored in 4 °C, rubber septa were used for closing the ends. DMA-coated capillaries were prepared as described by Wan et al. [7] and all coated capillaries were evaluated before used for electrophoresis as described by Williams and Vigh [32]. Uncoated capillaries were treated with 0.1 M HCl for 3 min and running buffer for 1 min, prior to each run.

If not stated otherwise, the following procedures were used: Electrophoresis was performed on a Hewlett-Packard ^{3D}CE system (Waldbronn, Germany), equipped with a diode array detector, an air-cooled capillary cartridge set to 22 °C and, in order to preserve the proteins, an external water bath (Grant LDT6G, Cambridge, UK) giving the vial carousel a temperature of 8 °C. Detection was with direct UV at 200 nm. Injection of samples was performed as follows: a pressure of 50 mbar was first applied for 5 s to the vial containing the marker, then to the vial containing the sample. Lastly, a small plug of buffer was introduced to the capillary using a pressure of 10 mbar for 5 s. A stock solution of phosphate buffer adjusted to pH 7.4 with NaOH was diluted prior to CE analysis to give a running buffer with a concentration of 0.1 M.

Quantitative binding data were estimated from measurements of peak appearance shifts in affinity capillary electrophoresis (ACE) at different ligand concentrations. Binding constants were estimated by non-linear curve fitting (one-site binding hyperbolas) by the GraphPadPrism software (v. 3.0) (San Diego, CA, USA) to data of $\Delta(1/t)$ as a function of ligand concentration. The $1/t$ value is a measure of μ the electrophoretic mobility when capillary dimensions, buffer conditions (except for ligand addition), current and field strengths are kept constant. The $\Delta(1/t)$ value is the difference in corrected inverse peak appearance times between experiment with and without added ligand, i.e. $\Delta(1/t) = (1/t - 1/t_r) - (1/t_0 - 1/t_{r0})$, where t is the peak appearance time, r denotes the reference peak (non-interacting

marker), and subscript 0 denotes the experiment without ligand added [3].

3. Results and discussion

3.1. Analytical recovery of $\beta_2$gpI

Purified $\beta_2$gpI at 1–2 mg/ml (18–36 μM) was not recoverable in CE experiments using neutral pH electrophoresis buffers and plain fused-silica capillaries (data not shown). A simple way to assess capillary adsorption problems in the analysis of specific proteins is to perform a running buffer pH scan. This will in some cases show that CE analysis is feasible at a pH value that is acceptable for subsequent binding studies. Thus, a CE-based study of antigen–antibody binding showed that not only would a slightly alkaline pH allow reproducible recovery of the antibody but would also be compatible with preserved antigen–antibody interactions [33]. At neutral to slightly alkaline pH $\beta_2$gpI has almost the same slow electrophoretic mobility as antibodies and therefore initially the analysis of the purified protein in bare silica capillaries was tested at different electrophoresis buffer pH values. However, pH values close to physiological pH were not compatible with the recovery of $\beta_2$gpI (data not shown).

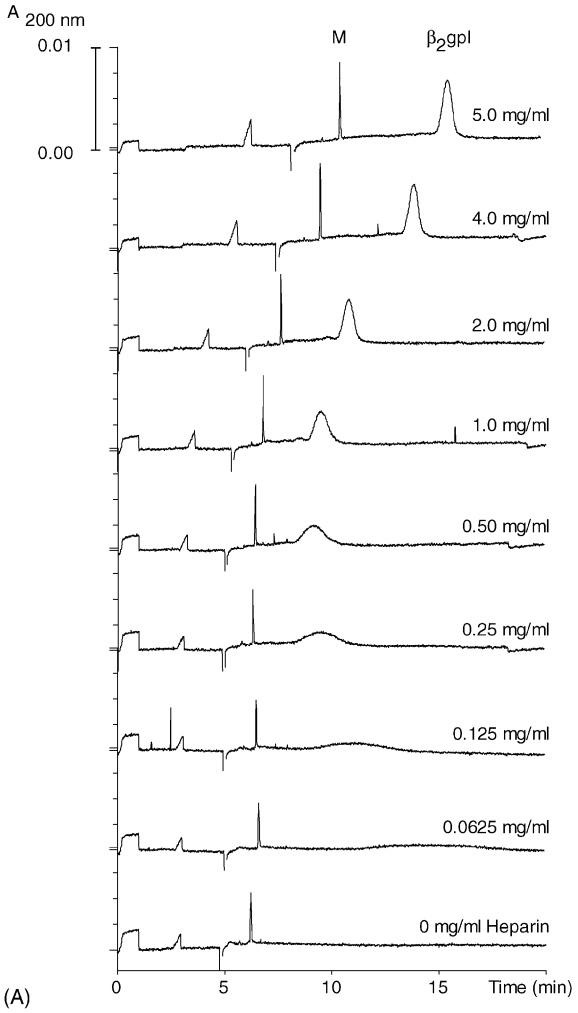
3.1.1. Mobilization using ligand addition to running buffers in coated and uncoated capillaries

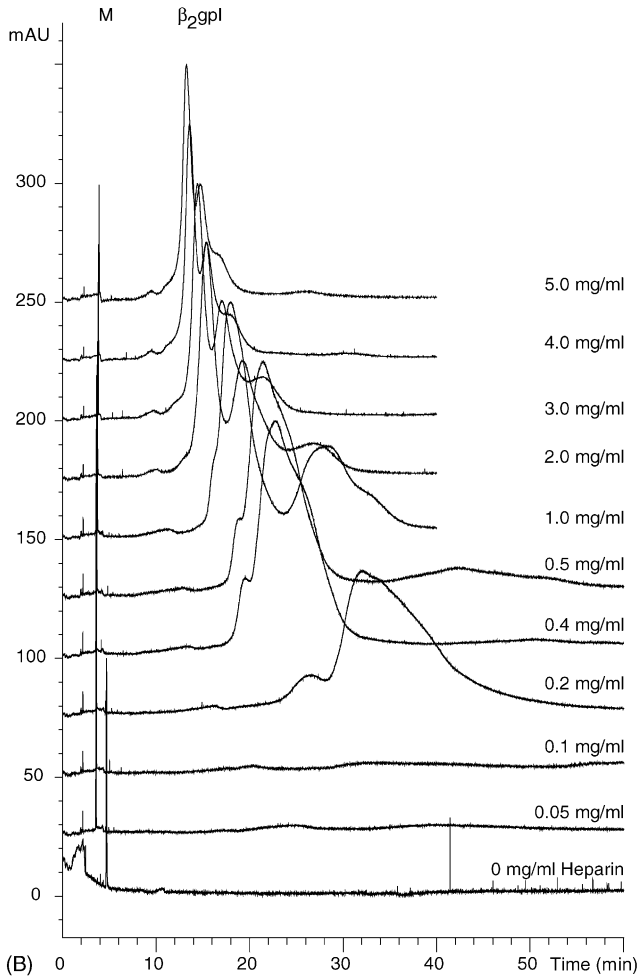
A more specific suppression of wall interactions may be achieved using a known ligand that confers a suitable charge to the analyte, e.g. a negative charge upon complex formation with basic analytes such as has been illustrated in the analysis of human lactoferrin where heparin was a necessary buffer additive [34]. $\beta_2$gpI is also a heparin binding protein and, accordingly, it is mobilized through inclusion of heparin in the electrophoresis buffer as found in preliminary experiments at pH 8.6 in uncoated capillaries and in a polyacrylamide-coated capillary as shown in Fig. 1. Here, the analyte peak using buffer conditions – where the peak is normally absent – begins to emerge when heparin is added to the running buffer at more than 0.1–0.2 mg/ml. However, in the uncoated capillary (Fig. 1A), somewhat counterintuitively, the $\beta_2$gpI peak appearance time is shortened at increasing heparin concentrations up to 0.5 mg/ml before it begins to be prolonged as would be expected when it interacts with a negatively charged ligand. Given that the mobility of $\beta_2$gpI is zero when binding to the negatively charged groups on the capillary wall, the liberation by a ligand (heparin) in solution will bring some molecules past the detector, i.e. molecules that have been eluted from the wall sites by complexation with the soluble heparin. With higher heparin concentrations the liberated fraction will be larger, but still $\beta_2$gpI will be engaged in interactions with both the wall and with the moving heparin molecules giving the pronounced broadening of the analyte peak at added heparin concentrations below 0.5–1.0 mg/ml. The migration shift begins to turn in the other direction when the interaction with heparin is the predominant complexation event (from about 1 mg/ml heparin additive). However, at the same time the electroosmotic flow (EOF) also begins to slow down probably because the highly negatively charged heparin additive attracts the buffer counter ions that otherwise cover the wall and cause the EOF. Thus, even though the $\beta_2$gpI peak in the experiments of Fig. 1A is recovered, the presence of interactions with soluble ligand as well as with wall charges in addition to concomitant changes in EOF make it difficult to use this approach to estimate binding parameters.

Other previously established methods [7,8] used to statically coat capillaries were also tested (e.g. Fig. 1B). Using acrylamide and dimethylacrylamide we found partial but inconsistent recovery of $\beta_2$gpI in the absence of buffer additives (data not shown). The reproducibility was poor and in the dimethylacrylamide (DMA)-coated capillaries $\beta_2$gpI was – as a rule – not recovered at all while plain acrylamide gave a better result (Fig. 1B). The reason for the bad recovery, especially in the DMA-coated capillaries, is not known but $\beta_2$gpI has a hydrophobic region exposed to the solvent [24,35] and has well-known lipid binding capabilities (cf. below) and thus has an affinity for hydrophobic surfaces. Accordingly, the higher adsorptivity of $\beta_2$gpI on the DMA-coating compared with acrylamide may be due to the more non-polar character of DMA. Again, mobilization of $\beta_2$gpI with heparin added to the running buffer in the acrylamide-coated capillaries made reproducible analyses possible (Fig. 1B). In contrast to the analysis in plain capillaries at pH 8.6, $\beta_2$gpI behaves as a heterogeneous molecule when mobilized in this way in the coated capillaries at pH 7.4 and the migration shifts are now unidirectional reflecting that $\beta_2$gpI spends more and more time as a complexed molecule during electrophoresis. This is observed as decreased appearance times since the analysis is run at reversed polarity. In DMA-coated capillaries essentially the same results were obtained (data not shown). The above experiments showed that $\beta_2$gpI could be recovered at neutral pH in coated capillaries but only reproducibly in the presence of added anionic ligand in the CE buffer. However, for use in ACE experiments any approach employing mobilization by addition of a known ligand is not optimal because several binding interactions subsequently will have to be analyzed.

3.1.2. Use of pH-hysteresis effect to counteract $\beta_2$gpI wall interactions in uncoated capillaries

Thus, we next tried to exploit the so-called pH-hysteresis effect to change the wall charge while maintaining a neutral running buffer pH. This avoids wall interactions at the same time as binding experiments are feasible and meaningful because no additional components are required to be present in the system. The pH-hysteresis effect has been useful for manipulations of EOF, e.g. in studies where it was important to get different peak appearance times without changing running buffer composition and field strength [26]. Lambert and Middleton [25] showed that the equilibration of the surface charge on the capillary wall is a relatively slow process


A 200 nm
0.01
0.00
M
β2gpI
5.0 mg/ml
4.0 mg/ml
2.0 mg/ml
1.0 mg/ml
0.50 mg/ml
0.25 mg/ml
0.125 mg/ml
0.0625 mg/ml
0 mg/ml Heparin
0
5
10
15
Time (min)
(A)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300
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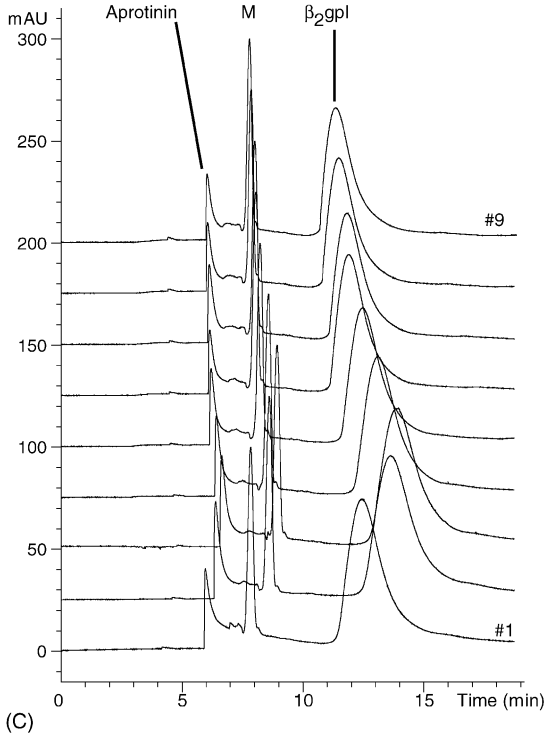
2.0 mg/ml
1.0 mg/ml
0.5 mg/ml
0.4 mg/ml
0.2 mg/ml
0.1 mg/ml
0.05 mg/ml
0 mg/ml Heparin
0
10
20
30
40
50
Time (min)
(B)


mAU
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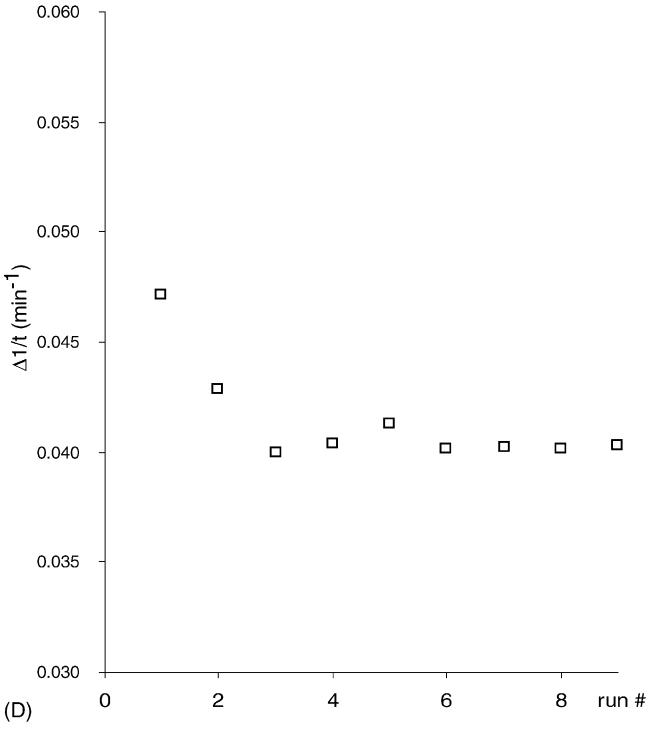
(C)


0.060
0.055
0.050
0.045
0.040
0.035
0.030
Δ1/t (min-1)
0
2
4
6
8
run #
(D)

and fewer deprotonated silanol groups are present after preconditioning at an acidic pH. Huang discussed a model of this effect and suggested that the formation of a porous gel layer near the silica surface affects the kinetic factors for the establishment of the equilibrium state for the silica–solution interface [36]. However, the pH-hysteresis phenomenon does not appear to have been previously utilized directly to combat wall-adsorption problems in CE analyses of proteins. We here used it to decrease analyte–wall interactions simply by protonating the silanol groups by an acidic pre-rinse instead of the routinely used basic pre-wash with NaOH.

This approach was successful in eliminating recovery problems in the CE analyses of $\beta_2$gpI, i.e. after washing the plain silica capillary with 0.1 M HCl prior to every run, reproducible recovery as shown in Fig. 1C and D was achieved. Peak areas and adjusted appearance times showed a variability of 13.6 and 5.7%, respectively, in nine consecutive runs. The migration times for both the neutral marker (DMSO) and the analyte showed a small variation for the first few runs, but these variations diminished with repeating analyses and thus it appears that the capillary is stabilized with increasing number of runs (Fig. 1D). After establishing the utility of the pH-hysteresis effect, the approach was next used for the CE-based analyses of $\beta_2$gpI binding to heparin and anionic liposomes.

3.2. Binding experiments with heparin

Binding studies with the negatively charged ligand heparin was performed by adding different amounts of the ligand to the electrophoresis run buffer. As expected, binding of $\beta_2$gpI to the highly negatively charged heparin caused a migration shift to a longer migration time compared with the neutral marker (Fig. 2). Due to the EOF, the net mobility was still toward the cathode. The analyte peak was split in three not fully separated peaks (labeled I–III in the figure) upon addition of heparin. This was also seen in experiments in coated capillaries (Fig. 1B). When the amount of heparin was increased the peaks first became more separated but the first appearing peak (labeled I in Fig. 2A) showed pronounced broadening even at relatively low concentrations of heparin and therefore quantitative binding data (that require determination of the peak appearance time) were difficult to obtain for that fraction. With more heparin added to the electrophoresis buffer, i.e. at concentrations above 3 mg/ml, the two last peaks (labeled II and III in Fig. 2A) started to co-migrate. To extract quantitative binding data the average values of $\Delta(1/t)$ (cf. Section 2.3) from three replicate runs for peaks II and III in Fig. 2A were plotted against the concentration of heparin (Fig. 2B). The analysis is restricted to data from analyses below 3 mg/ml heparin. A one-binding site hyperbola function was fitted ($R^2 > 0.9$ in both cases) to each of the data set and yielded dissociation constants of 0.73 mg/ml (49 μM) and 0.23 mg/ml (15 μM) for peaks II and III, respectively. These binding constant values are estimates because of the uncertainties in defining the precise peak positions and because the binding isotherms are covered only partly. Also, a number of assumptions including the presence of a 1:1 stoichiometry of binding are made to perform these quantitative estimates [3]. The curve fits of Fig. 2B do not contradict this assumption. Thus, the results suggest that the peak III component binds heparin approximately three times stronger than peak II. Also, the apparent fusion of the binding curves above 2.5 mg/ml heparin suggests the same binding stoichiometry of the two fractions represented by the two peaks. Using lower ionic strength electrophoresis buffer, the binding curves, while still showing the same relative differences in affinity, were left-shifted, i.e. indicating that the heparin binding by $\beta_2$gpI predominantly involves electrostatic interactions (data not shown).

The multiple peaks of apparently differently binding $\beta_2$gpI-species that were resolved in the binding experiments with heparin could be caused by several factors. Obviously, the data indicate that there is more than one type of binding sites present in the protein solution with differences in affinity. The retardation peaks do not represent protein–ligand complexes but differently retarded protein peaks that represent analyte species that reversibly interact differently with the heparin present in the CE buffer. This could be due to contaminating proteins, different conformations of $\beta_2$gpI and/or structural variants of $\beta_2$gpI that are not resolved without increases in selectivity. Other explanations could be that some $\beta_2$gpI populations are only recovered at high ligand concentrations. Also, heparin itself may be heterogeneous with different binding to $\beta_2$gpI of subfractions of this ligand. Finally $\beta_2$gpI exhibits glycosylation microheterogeneity [37] and different glycoforms may have different ligand binding characteristics. It is not simple to assess binding specificity

Fig. 1. Recovery of $\beta_2$gpI. (A and B): Mobilization of $\beta_2$gpI by affinity-CE with bovine lung heparin added to the electrophoresis buffer. (A) Electropherograms of $\beta_2$gpI at 0.45 mg/ml in 4× diluted phosphate buffered saline (PBS) with 0.07 mg/ml marker peptide (M). Injection: 6 s at 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa). Capillary: uncoated fused silica, 50 cm to detector (57 cm total length); voltage 15 kV; temperature 20 °C. Electrophoresis buffer: 0.13 M Tris base, 0.5 M glycine, pH 8.6 with added bovine lung heparin at the mg/ml concentrations given in the figure. CE instrument: Beckman PACE System 2050 with the System Gold software. (B) Electropherograms of 5 mg/ml $\beta_2$gpI in 2× diluted PBS; marker (M), 300 μM phthalic acid. Injection: marker, 5 s at 50 mbar; sample, 5 s at 50 mbar; buffer, 5 s at 10 mbar. CE instrument: Hewlett-Packard ^{3D}CE system. Capillary: acrylamide coated, 32 cm to detector (40 cm total length); constant current conditions: −120 μA; temperature 22 °C. Electrophoresis buffer: 0.1 M phosphate pH 7.4 with added bovine lung heparin in the mg/ml concentrations given in the figure. (C) Electropherograms of 5 mg/ml $\beta_2$gpI in 2× diluted PBS; markers, 0.2 mg/ml aprotinin and 1% (v/v) DMSO (M). Injection and instrument as in (B). Capillary: uncoated fused silica pre-treated with 0.1 M HCl for 3 min prior to each run, 32 cm to detector (40 cm total length); voltage 15 kV; temperature 22 °C. Electrophoresis buffer: 0.1 M phosphate pH 7.4. (D) Plot of $\Delta(1/t)$ (difference in the inverse appearance time of marker and analyte) for $\beta_2$gpI in (C) against run number.

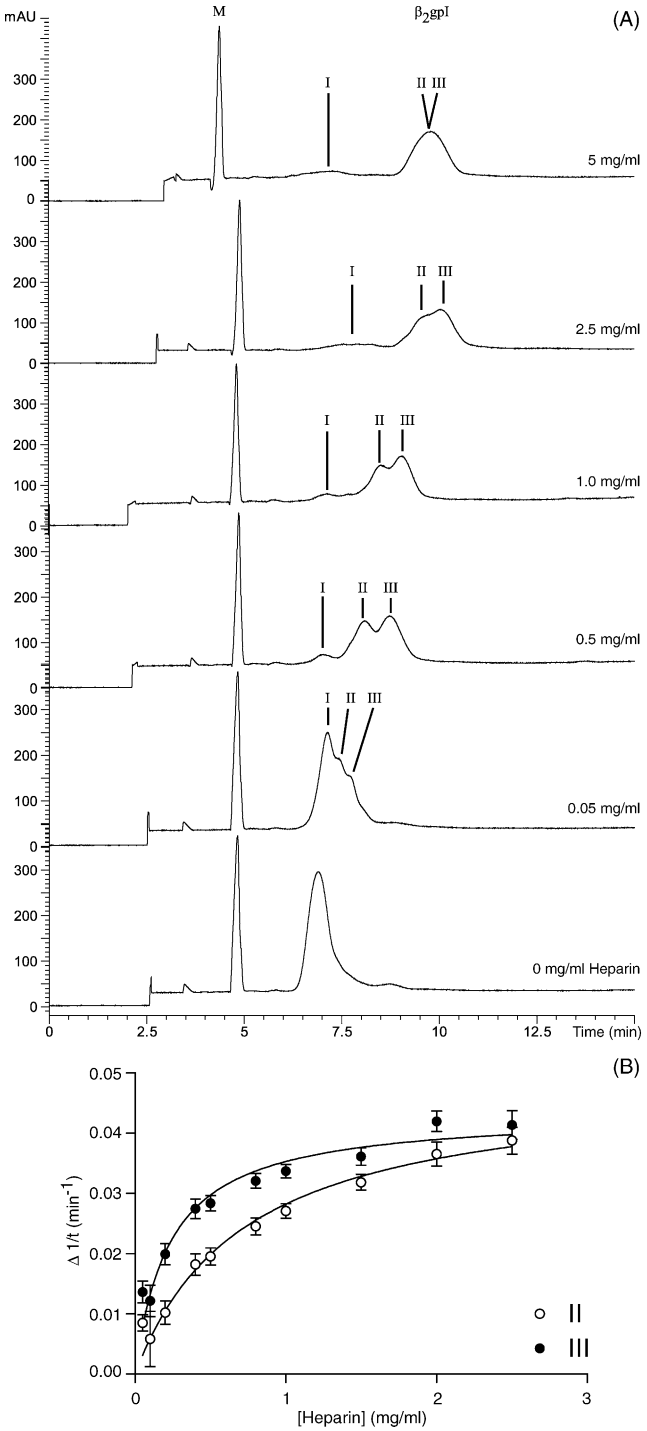


Fig. 2. Interaction of β2gpI with heparin. (A) Electropherograms of 5 mg/ml $\beta_2$gpI in 2× diluted PBS; marker (M), 1% (v/v) DMSO. Injection as in Fig. 1B. Capillary as in Fig. 1C; constant current conditions: 120 μA; temperature 22 °C. Electrophoresis buffer: 0.1 M phosphate pH 7.4 with added bovine lung heparin in the mg/ml concentrations given in the figure. Fractions of $\beta_2$gpI (labeled I–III) were resolved. (B) Graph showing $\Delta(1/t)$ (average + S.D. of triplicate experiments) as a function of heparin concentration for peaks II and III (see A) up to 2.5 mg/ml added ligand. Non-linear curve fitting using a one binding site hyperbola as a model gave the binding isotherms shown ($R^2 > 0.9$ for both).

by adding an anionic non-binder since $\beta_2$gpI interacts with a broad class of anionic compounds. Supplementary studies, e.g. with a mass spectrometer as detector and using deglycosylated molecules as analytes, will be necessary to understand the causes of the splitting of the peaks. However, the results illustrate the unique capability of CE to fractionate heterogeneous analyte species at the same time as qualitatively and quantitatively probing for their ligand binding characteristics.

3.3. Binding experiments with phospholipids

Specific anionic phospholipids [phosphatidylserine (PS)] exposed on apoptotic cells are ligands for $\beta_2$gpI while other phospholipids have less affinity [10,19,23,38]. We explored the possibility of using the CE approach for detecting and characterizing such interactions. In aqueous solutions hydrophobic interactions among the lipid parts of phospholipids drive the formation of micelles, lipid bilayers, or spherical vesicles (liposomes). Liposomes are the most stable form in aqueous solutions and we therefore used liposome preparations as buffer additives in an ACE approach with acid prewash to test for $\beta_2$gpI binding. The procedure described by Wiedmer and co-workers [27–30] was used to generate large unilamellar vesicles composed of different ratios of the uncharged phospholipid phosphatidylcholine (POPC) and the anionic phospholipid PS. The amount of the anionic phospholipid was varied between 0 and 30 mol%. A higher content of anionic phospholipid could cause the vesicle to undergo a phase transition from lamellar to non-lamellar phase [29] and was therefore not used. The buffer in which the lipids were dissolved was the same as the electrophoresis buffer in order to retain the osmotic balance between the interior and the exterior of the liposome, as described in [29].

When liposomes were simply added to the running buffer the baseline became very unstable and the analyte peak could not be distinguished from the background (data not shown). Liposomes are colloidal particles that scatter light and thus interfere with the UV detection. To avoid that, the partial filling technique was used, i.e. injecting the ligand as a discrete sample plug [39,40].

The liposomes containing PS are negatively charged due to the content of anionic phospholipid (cf. also below) and their electrophoretic mobility is toward the anode, while $\beta_2$gpI has a lower anodic mobility and therefore emerges faster. Accordingly, to ensure mixing of sample and liposome zones, the liposomes were injected as a plug in front of the sample according to the scheme outlined in Fig. 3B. Even when liposomes were only partially filling the capillary, however, all peaks disappeared after a few runs. After a couple of runs without liposomes present, the peaks reappeared. The current and voltage were stable during the entire analysis, thus the problem was suspected to be of a chemical character and with a washing step consisting of a 10 min flush at 4 bar with 0.5 M HCl the problem was resolved. In a recent publication, Hautala et al. [27] described a method for coating capillaries with phospholipids. They studied the influence of the buffer

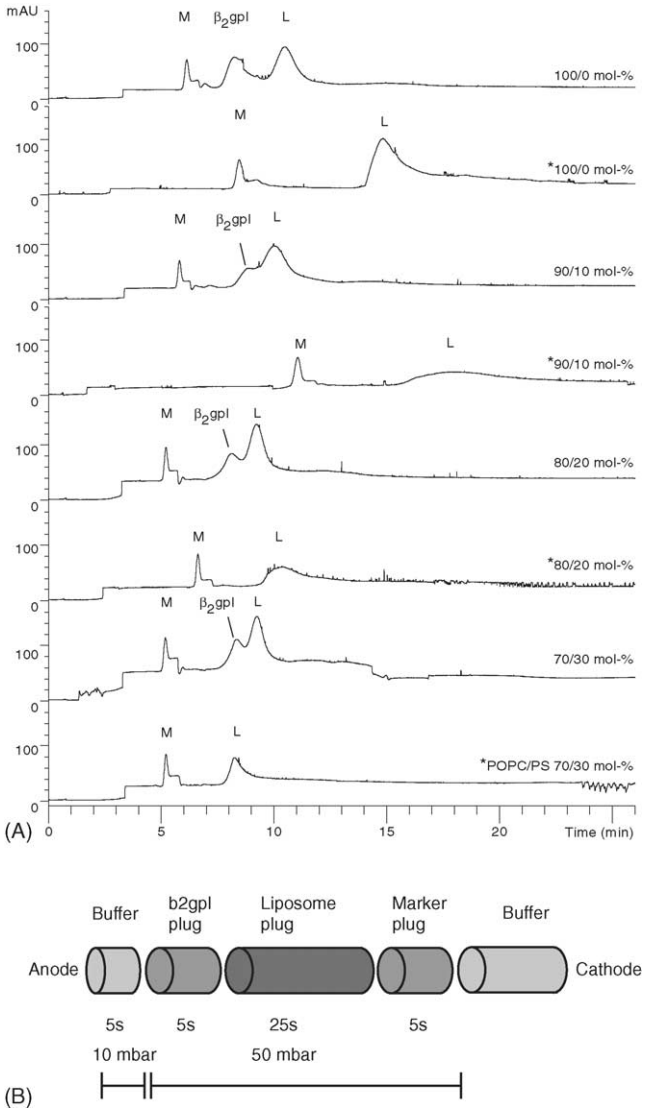


Fig. 3. Interaction of $\beta_2$gpI with phospholipids using partial filling ACE. (A) Electropherograms of 1 mg/ml $\beta_2$gpI in 10× diluted PBS; marker (M), 0.1% (v/v) DMSO; liposomes (L), 4 mM total lipid concentration in compositions given in the figure. Injection: marker, 5 s at 50 mbar; liposome, 25 s at 50 mbar; sample, 5 s at 50 mbar; buffer, 5 s at 10 mbar. Capillary as in Fig. 1C treated with 0.5 M HCl for 10 min at 4 bar between runs; constant current conditions: 120 μA; temperature 22 °C. Electrophoresis buffer: 0.1 M phosphate pH 7.4; (*) denotes control runs, i.e. buffer was injected instead of the $\beta_2$gpI sample. (B) Injection scheme for the runs in (A).

solution on the interaction between liposomes and the capillary wall and found that interaction occurred, to some extent, with all the studied buffers, including phosphate. Thus, we suspect that the liposomes adhere to the capillary and create a phospholipid bilayer at the wall yielding a dynamic coating with phosphate as linker. Therefore, no or only a small EOF will be present and the neutral marker will not be detected. Also, $\beta_2$gpI will bind to the immobilized phospholipid bilayer and, as a consequence, the analyte will not be detected. Washing the capillary at high pressure with a relatively strong acid, ensures the detachment of the liposomes stuck on the wall and enables subsequent analyses.

Fig. 3A shows the electropherograms obtained from the binding studies of $\beta_2$gpI and liposomes when the partial filling technique was used with 0.5 M HCl pre-wash. It was observed that even the zwitterionic phospholipid, POPC without any PS present had an electrophoretic mobility toward the anode (upper panel, Fig. 3A), i.e. the peak (L) representing the liposomes is seen after the sample peak. Theoretically, zwitterionic phospholipids should form a liposome with a neutral overall charge and therefore the liposome should have no electrophoretic mobility. However, liposomes composed by zwitterionic phospholipids have been found to exhibit non-zero mobilities in the presence of an external electrical field [29,30,41,42]. Makino et al. [42] measured the zeta-potential of liposomes composed of different zwitterionic phospholipids dispersed in different ionic strength buffers and explained this phenomenon as originating from the orientation of the lipid head group in the liposome. Pysher and Hayes [41] suggested that liposomal deformation and field-induced polarization may occur during electrophoresis.

Different migration patterns were obtained for $\beta_2$gpI in the presence of plugs of same total concentrations but different compositions of the liposomes. In the absence of a $\beta_2$gpI sample plug, however, it was observed that different liposome plug compositions by themselves had vastly different (differing more than 5 min) electroosmotic flows which were normalized in the presence of a $\beta_2$gpI sample. Thus, even though the data indicates retardation of the $\beta_2$gpI peak in the presence of anionic liposomes (indicating the affinity of $\beta_2$gpI for the PS-containing liposomes) the migration patterns are too complicated to reliably extract quantitative binding data. Experiments using a fixed composition but different total concentrations of liposomes but may resolve these issues. For its general use in CE analysis of proteins a more thorough characterization of the method will be forthcoming.

4. Conclusions

In the development of suitable conditions for analysis at neutral pH of the basic protein β_2-glycoprotein I we found the pH hysteresis behavior of fused silica surface useful since the more protonated surface after an acid pre-wash counteracted protein adsorption efficiently. An acidic pre-wash was a simple procedure that made estimates of binding interactions of $\beta_2$gpI with heparin and liposomes by affinity CE feasible.

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