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Lipid/Polydiacetylene Films for Colorimetric Protein Surface-Charge Analysis

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The distribution and organization of charges on a protein surface are fundamental properties which affect protein functions and interactions. We demonstrate a new approach for protein surface-charge analysis through modulating protein interactions with chromatic lipid/polydiacetylene (PDA) films. We show that visible and easily quantifiable blue–red transitions, induced on the film surface through electrostatic interactions between the negatively charged PDA and positive soluble species, constitute an effective means for characterizing protein surface charge. Specifically, protein–film interactions can be significantly modulated by complexation between the tested macromolecules and lipid-embedded multivalent calixarene ligands displaying charged residues, making possible protein discrimination based upon the abundance and organization of surface charge. The lipid/PDA film system, in conjunction with the calixarene-derived ligands, facilitates characterization of protein surface charges and identification of anomalous protein electrostatic properties.

The sites and organization of surface charges have profound significance in determining protein properties and functions, interactions with other proteins and biomolecules, and their the overall stability and conformational changes.^{1–4} Charged surfaces on globular proteins generally constitute the preferred contact areas involved in protein–protein interactions,^{1,5,6} protein binding to surfaces,^{7–9} and as docking sites for biological and synthetic ligands.^{1,10,11}

Recent studies have exploited protein surface charge for macromolecular binding and recognition by artificial ligands^{11–13} and for nonspecific Coulomb attraction by amphiphiles¹⁴ or polymers.¹⁵ Multivalent ligands comprising calixarene scaffolds, in particular, have gained attention as vehicles for binding proteins through electrostatic attraction between the protein and charged residues within the artificial ligands.^{16–18} Calixarene-based multivalent ligands exhibit important advantages for macromolecular binding, particularly the availability of several reactive positions for attachment of recognition elements, and the overall stability conferred by the calixarene scaffolding.^{16,19} Calixarene-based ligands generally display high affinity to macromolecules, ascribed to the multivalent nature and the combination of preorientation and flexibility of the binding residues.^{16–18}

Calixarene-based multivalent ligands decorated with charged residues (Figure 1) have been previously used in our laboratories for biomolecular sensing applications.^{17,20} Published studies have focused on incorporation of the ligands within hydrophobic environments, including fatty-acid monolayers¹⁷ and biomimetic membrane vesicles,²⁰ and subsequent analysis of biophysical transformations induced by protein binding. The measurable quantities evaluated in these studies were pressure/surface-area (p/A) changes in monolayers at the air/water interface¹⁷ or chromatic transitions in lipid/polydiacetylene vesicles.²⁰ In these investigations, the modular organization of the calixarene-based ligands constituted an important structural feature: the protruding charged residues attained multicontact binding to the macromol-

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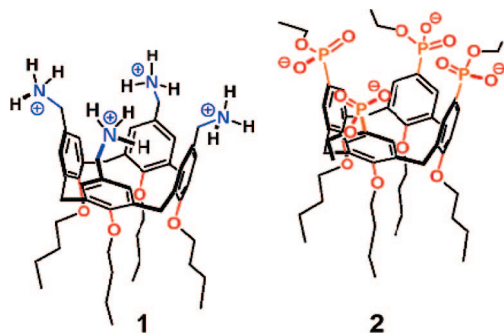


Figure 1. Calixarene-based ligands: **1**, calixarene benzylammonium, $pK_1 = 9.5$; **2**, calixarene tetraphosphonate, $pK_1 = 1.8$.

ecules, while the calixarene moieties were essential for anchoring the molecular host within the hydrophobic scaffoldings (monolayers or vesicles).

In this study we demonstrate a new approach for characterizing and exploiting protein surface charge through induction of colorimetric transformations in glass-supported phospholipid/polydiacetylene (PDA) films induced by water-soluble globular proteins. In particular, the charged calixarene-based ligands **1** and **2** were employed here as a vehicle for modulating protein–film interactions. PDA-based assemblies, including small vesicles,^{21–23} giant vesicles,²⁴ labeled cells,²⁵ thin films,^{26–28} and varied composite materials^{29–33} have been shown to exhibit versatile molecular sensing properties. PDA-based sensing schemes exploit the unique chromatic properties of the polymer: polymerized PDA appears as an intense blue due to the conjugated ene-yne framework³⁴ and undergoes dramatic blue–red changes due to a structural transition in the conjugated backbone that is induced by external structural perturbations, primarily through binding of varied amphiphilic analytes.^{21,35,36}

The experimental setup facilitates interactions between positively charged species with the PDA domains in the films, resulting in intense and easily quantified blue–red transitions. Amphiphilic complexes between the calixarene ligands and proteins exhibiting surface charges, on the other hand, did not give rise to color changes due to their targeting to the phospholipid domains in the films. Overall, the lipid/PDA films provide

an effective vehicle for analysis of protein surface-charge and its reorganization following protein interactions in solutions.

MATERIALS AND METHODS

Materials. The monomeric diacetylene 10,12-tricosadiynoic acid was purchased from Alfa Aesar (Karlsruhe, Germany). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Sigma. The phospholipids 2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1- α -phosphatidylserine, and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL). The peptides and proteins glycoprotein, amyloglucosidase, trypsin inhibitor, β -lactoglobulin A, proteinase k, trypsinogen, histone, lysozyme, pepsin, bovine serum albumin, trypsin, cytochrome C, and polyarginine were purchased from Sigma. All the proteins were dissolved in water. The oligonucleotide (5'-CTG-ACC-CTG-TCA-GAC-TGT-GGG-GAC-AAG-GTG-3') was purchased from Integrated DNA Technologies (Coralville, IA). Chloroform (CHCl_3) was HPLC grade (Frutarom Ltd.).

Preparation of Glass-Supported Lipid/PDA Films. The monomeric diacetylene 10,12-tricosadiynoic acid was purified by dissolving the powder in chloroform, filtering through a 0.45 μm nylon filter, and evaporating the solvent. DMPC and diacetylene were dissolved in chloroform and mixed at a total concentration of 2 mM (mole ratio = 9:1 between diacetylene and phospholipid). The water subphase used in the Langmuir trough was doubly purified by a Barnstead D7382 water purification system (Barnstead Thermolyne Corporation, IA), producing distilled water having 18.3 M Ω resistance.

All DMPC/diacetylene films were prepared in a computerized Langmuir trough manufactured by NIMA (model 312D, Nima Technology Ltd., Coventry, U.K.). The experiments were carried out at 10 $^\circ\text{C}$. The surface pressure was monitored using a 1-cm-wide filter paper as a Wilhelmy plate. For each experiment, 40 μL of the lipid/diacetylene solution was spread on the water subphase (pH 6.3). Compression started after solvent evaporation (15 min) and was carried out at a constant barrier speed of 12 $\text{cm}^2 \text{min}^{-1}$. The films were compressed to 18 mN/m and then allowed to equilibrate at a constant surface pressure for 5 min. The films were then transferred onto glass slides by the horizontal touch method (Langmuir–Schaefer method) and then irradiated at 254 nm to polymerize the diacetylene (resulting in a blue appearance of the films).

Colorimetric Analysis. Aliquots of the tested solutions were placed on the surfaces of the films and incubated at 37 $^\circ\text{C}$ for several minutes. The films were sealed in a Petri dish to eliminate evaporation. Following incubation, the drops were removed and the dry films were scanned on an Epson 4990 photo scanner to produce high-resolution RGB images. The films were placed in a special film adaptor and scanned in the transmitted mode at an optical resolution 2400 dpi and color depth of 24 bit.

Digital colorimetric analysis (DCA) was carried out after cropping the sample spots in the scanned images. Color change values were calculated using MATLAB software as detailed previously.³⁷ Briefly, the extent of red intensity in each pixel was calculated as the red chromaticity level (r)³⁸

$$r = \frac{R}{R + G + B}$$

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where *R* (red), *G* (green), and *B* (blue) are the three primary color components. For a defined surface area within a lipid/PDA film, we classify a quantitative parameter denoted red chromaticity shift (RCS) that represents the total blue-red transformations of the pixels encompassed in the area:

$$\text{RCS} = \frac{r_{\text{sample}} - r_0}{r_{\text{max}} - r_0} \times 100\%$$

where r_{sample} is the average red chromaticity level of all pixels in the scanned surface, r_0 is the average red level calculated in a blank surface (blue film initially prepared prior to treatment), and r_{max} is the average red chromaticity level of the maximal blue–red transition, an area of the film in which the most pronounced blue–red transition was induced (positive control, usually achieved by incubation with an organic solvent such as ethanol). In essence, RCS is the normalized change in the red chromaticity level within the film surface on which the tested sample was deposited.

The net colorimetric effect was calculated as

$$\text{net RCS} = \text{RCS}_1 - \text{RCS}_0$$

where RCS_1 (%) is the RCS after addition of the pure protein or the protein–calixarene complex to the DMPC/PDA films while RCS_0 (%) is the red chromaticity shift obtained after addition of water (in the case where a pure protein was added) or the calixarene by itself, respectively.

Raman Experiments. The Raman measurements were conducted on a Jobin-Yvon LabRam HR 800 micro-Raman system, equipped with a liquid-N₂-cooled detector. The excitation source was a He–Ne laser (633 nm) with a power of 6 mW on the sample. The laser was focused with a x50 long-focal-length objective to a spot of about 3 μm . Measurements were taken with the 600 g mm^{−1} grating and a confocal microscope with a 100 μm hole and a typical exposure time of 1 s.

Tryptophan Fluorescence Excitation. Samples for the tryptophan excitation experiments were placed on the surfaces of the polymerized films followed by drying through incubation at 37 °C. The tryptophan intrinsic excitation was measured on an Edinburgh Co. (Edinburgh, Scotland, U.K.) FL920 spectrofluorimeter, using excitation at 280 nm and emission at 360 nm. The glass films were placed in a special holder for surface measurements. Changes in tryptophan intrinsic excitation were measured again after the films were washed with distilled water and dried gently with filter paper.

Adsorption Onto the Lipid/Water Interface. Adsorption experiments were carried out at 25 °C using a Nima 312D Teflon trough. Chloroform-dissolved DMPC/diacetylene solution (7:3 mole ratio) was spread over the clean air/water interface of the dipping well (total volume of 50 cm³) and allowed to equilibrate for 15 min reaching the desired initial surface pressure. Aliquots (50 μL) of the tested solutions (dissolved in water) were then injected through a short vertical tube close to a magnet stirrer approximately 2 cm underneath the DMPC/diacetylene mono-

layer. The adsorption isotherms ($\Delta\pi$ -time) of the samples at a constant monolayer interface area (25 cm²) were monitored using the Wilhelmy plate.

Lipid Vesicles. Vesicles comprising DOPC, DOPC/L- α -phosphatidylserine, and DOPC/ cardiolipin (1:1 molar ratios) for analysis of protein interactions were prepared at a concentration of 1 mM. The lipids were dried together in vacuo. Following evaporation, distilled water was added and the suspension was probe-sonicated in an ice bath.

RESULTS

Principles of Protein Surface Charge Analysis Using Colorimetric Films. The colorimetric approach we have developed is shown schematically in Figure 2. The sensing platform was comprised of blue (polymerized) DMPC/PDA films assembled at the air/water interface and transferred onto a solid glass substrate.^{27,37} The thrust of the new methodology is the observation that, on a microscopic level, the films consist of distinct domains of PDA (which exhibit negatively charged carboxylate headgroups and zwitterionic DMPC monolayers, which are highly amphiphilic).^{27,28}

The formation of interspersed lipid and polymer domains in the films entails an interesting interplay between two competing interactions of water-soluble molecules with the film surface. On the one hand, positively charged molecules (either a protein or the positively charged ligand **1** employed in this study) are attracted to the negatively charged PDA headgroups, thereby giving rise to structural disruption of the pendant side-chains of the polymer and the corresponding blue–red transformation²⁶ (Figure 2A). On the other hand, complexes formed between charged-proteins and oppositely charged calixarene-based ligands exhibit enhanced amphiphilic properties,¹² thus experience high affinity to the lipid domains in the mixed films rather than the PDA, and in consequence induce significantly lower color changes on the film surface (Figure 2B).

Figure 3 depicts a representative colorimetric experiment and the quantitative analysis performed. Figure 3A features a scanned image of a blue DMPC/PDA film surface after short incubation with proteins, calixarenes, and protein–calixarene complexes. The reddish spots on the left side of the film reflect the pronounced blue–red transformations induced following interactions of the film surface with the positively charged ligand **1** (Figure 3A,i) and the positively charged protein histone (Figure 3A,iv). However, these blue–red transitions were greatly diminished when **1** was preincubated with amyloglucosidase, a highly acidic protein (Figure 3A,ii) or when histone was pre-incubated with the negative calixarene **2** (Figure 3A,v). Both the acidic protein amyloglucosidase (Figure 3A,iii) and the negatively charged ligand **2** (Figure 3A,vi) did not interact with the film and yielded negligible blue–red transitions.

The bar diagram in Figure 3B depicts the digital colorimetric analysis of the film image shown in Figure 3A (see Materials and Methods).³⁷ The diagram in Figure 3B provides the quantitative description for the colorimetric film transformations. Specifically, the red spots formed after interactions of **1** (Figure 3A,i) or histone (Figure 3A,iv) translate into the high % RCS values depicted in Figure 3B, while preincubation of amyloglucosidase with **1** (Figure 3A,ii), or of histone with **2** (Figure 3A,v) gave

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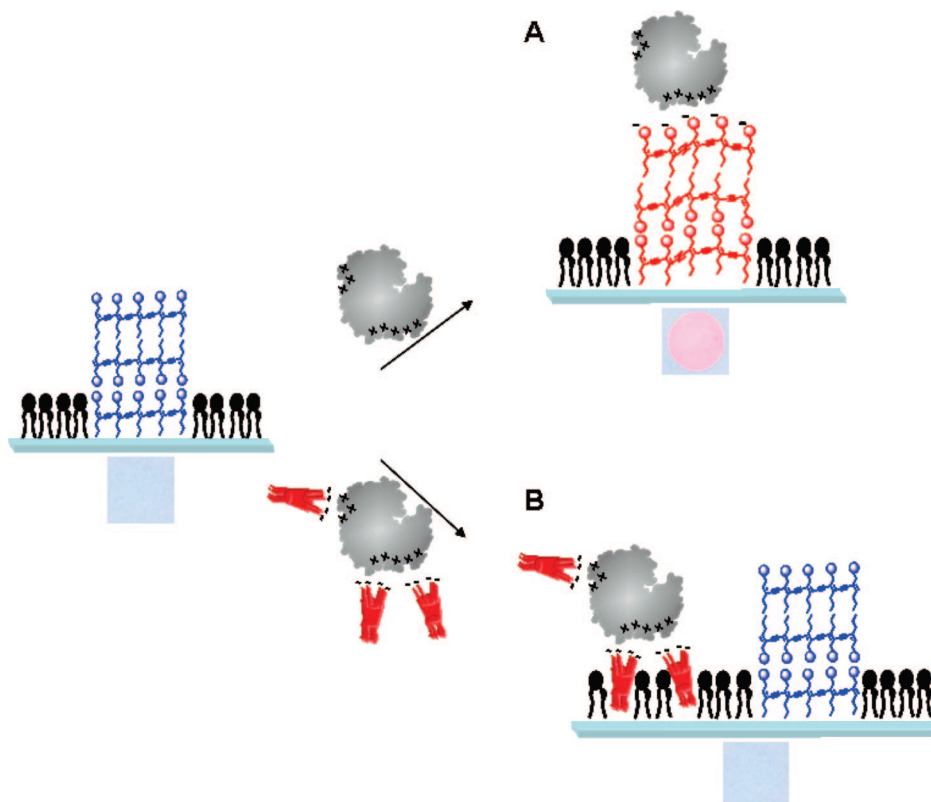


Figure 2. Protein–ligand interactions with lipid/PDA films. The glass-supported film comprising lipids (black) and PDA (blue) initially appears blue (scanned image shown). Two pathways are then observed: (A) positively charged proteins electrostatically bind to PDA, which in consequence undergoes a blue–red transformation and (B) neutralized, amphiphilic complexes formed between charged protein and the oppositely charged ligands are targeted to the lipid domains rather than the PDA; the film remains blue.

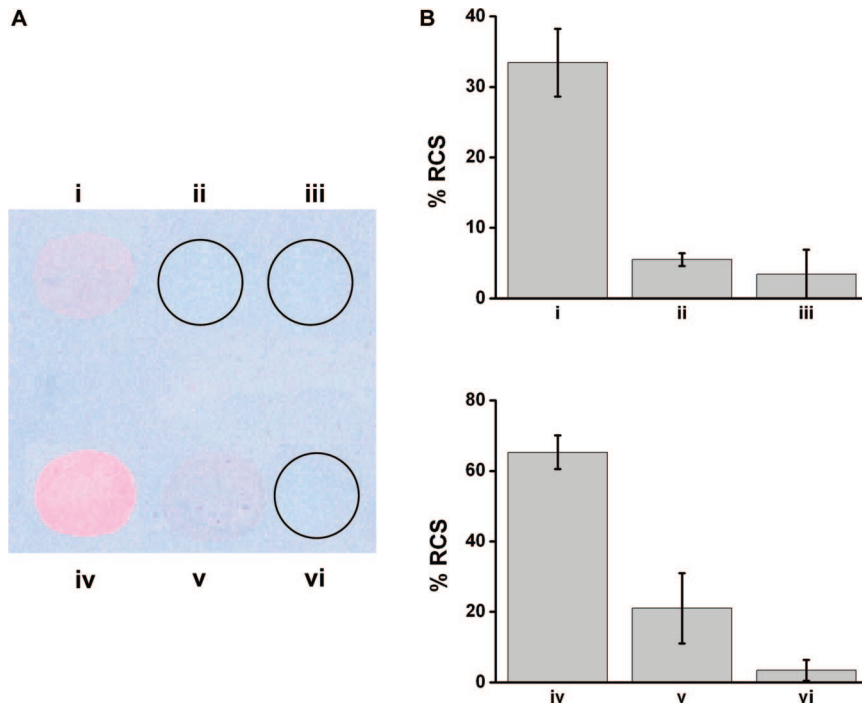


Figure 3. Color transitions induced by proteins and the calixarene-based ligands in a DMPC/PDA film. (A) Scanned film surface: (i) calixarene **1** ($2.5 \mu\text{M}$); (ii) **1** + amyloglucosidase ($66 \mu\text{M}$); (iii) amyloglucosidase ($66 \mu\text{M}$); (iv) histone ($66 \mu\text{M}$); (iv) histone + **2** ($22 \mu\text{M}$); (vi) calixarene **2**. (B) Quantitative DCA analysis of the scanned film in part A.

rise to significantly lower % RCS (Figure 3B). Similarly, the bluish spots in the film areas in which the negatively charged species were placed correspond to the negligible % RCS values (Figure

3B). It should be also emphasized that background effects of temperature or water upon the film color were insignificant (see Supporting Information).

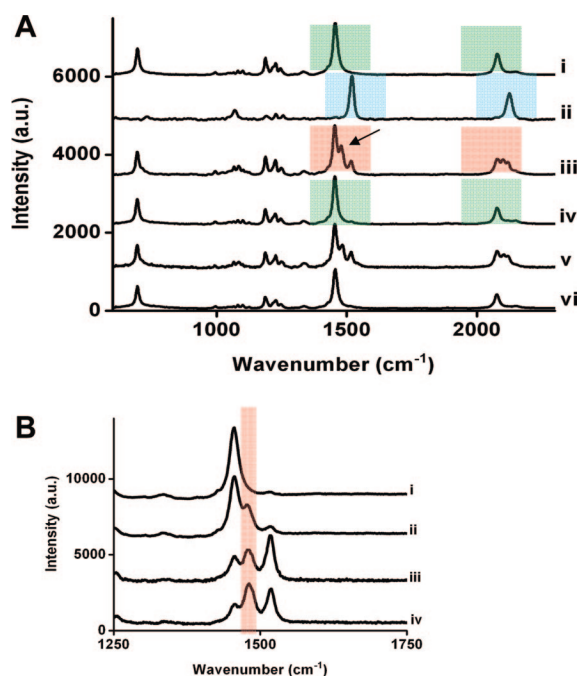


Figure 4. Raman spectroscopy of glass-supported DMPC/PDA films. (A) Entire spectral region: (i) polymerized blue DMPC/PDA film (1:9 mole ratio); (ii) film after 70 °C heating; (iii) addition of histone (66 μ M), the arrow points to the new peak at 1478 cm^{-1} ; (iv) addition of histone (66 μ M) preincubated with **2** (22 μ M); v. addition of ligand **1** (2.5 μ M); vi. addition of ligand **1** (2.5 μ M) preincubated with amyloglucosidase (66 μ M). The color shades highlight the spectral changes associated with the color transitions. (B) Expanded spectral area around 1500 cm^{-1} : blue DMPC/PDA film after addition of histone-**2** complex at different mole ratios (i) 3:1, (ii): 10:1, (iii): 20:1, and (iv): 30:1. Concentration of **2** was 22 μ M. The red shade emphasizes the 1478 cm^{-1} peak ascribed to electrostatic interaction with PDA, see text.

Molecular Characterization. Several analytical techniques have been applied to corroborate the model depicted in Figure 2 and to further probe film interactions of the proteins and the putative protein-calixarene complexes (Figures 4 and 5). Circular dichroism data (Supporting Information) confirm that stable complexes are formed between charged proteins and the calixarenes exhibiting opposite charges. Figure 4 presents the Raman analysis of the effects produced by direct interactions between DMPC/PDA films and proteins or protein-calixarene complexes. The Raman spectra of the polymerized blue DMPC/PDA film (Figure 4A,i) and the heat-transformed red film (Figure 4A,ii) highlight the structural changes associated with the blue-red transformation, particularly in the carbon-carbon double bond stretch around 1500 cm^{-1} and triple bond at approximately 2100 cm^{-1} (blue labels in Figure 4A,ii).

Different from the heat-induced spectrum, the Raman analysis of the DMPC/PDA films recorded after addition of proteins, ligands, and protein-ligand complexes, respectively, points to distinct structural effects associated with the observed color transformations. Figure 4A,iii depicts the spectrum of a DMPC/PDA film to which histone was added. Significantly, even though the blue film turned intense red following addition of this highly basic protein (scanned image in Figure 3A, iv), the Raman

spectrum of the film (Figure 4A,iii) was different than the heat-treated red film (Figure 4A,ii). In particular, note the emergence of a histone-induced signal at 1478 cm^{-1} (red labels in Figure 4A,iii), compared with the heat-induced peak at 1520 cm^{-1} . Importantly, the appearance of a peak at 1478 cm^{-1} following colorimetric transformations of PDA-based films is a new spectral feature which has not been reported before in Raman studies of polydiacetylene systems. This Raman peak most likely corresponds to an intermediate phase between the blue and heat-induced red configurations of PDA, corresponding to electrostatic binding between the negatively charged pendant side-chains and the bound positively charged protein guest molecules. Indeed, preincubation of the calixarene-tetraphosphonate ligand **2** with histone eliminated the Raman peak at 1478 cm^{-1} (Figure 4A,iv, green label). Similar phenomena were observed in the case of complex formation between the positive benzylammonium-calixarene **1** and the acidic protein amyloglucosidase (Figure 4A,v,vi): the peak at 1478 cm^{-1} was clearly apparent after incubation of **1** on the film surface (Figure 4A,v); however, it was abolished after preincubation of **1** with amyloglucosidase (Figure 4A,vi).

Figure 4B focuses on the Raman spectral region at around 1500 cm^{-1} and demonstrates the direct relationship between the intensity of the peak at 1478 cm^{-1} and the relative abundance of free histone vs the histone-**2** complex. The Raman spectra in Figure 4B show that the intensity of the signal at 1478 cm^{-1} directly correlates with the mole ratio between histone and ligand **2**. Indeed, the observation of a single Raman peak corresponding to blue PDA in Figure 4B,i indicates that at a 3:1 ratio (histone/**2**), almost no protein molecules were electrostatically bound to the PDA. The signal at 1478 cm^{-1} , however, starts appearing in a solution containing a histone/**2** ratio of 10:1 (Figure 4B,ii), in which an excess of free histone most likely emerges. This Raman peak becomes more pronounced at higher ratios between histone and the negatively charged ligand (Figure 4B,iii,iv), consistent with a greater abundance of noncomplexed histone interacting with the negatively charged PDA. It should be emphasized that the absolute concentration of histone was gradually increased from Figure 4B,i to Figure 4B,iv, giving rise to the more intense signal at 1520 cm^{-1} (compared to the 1478 cm^{-1} peak).

While Figure 4 confirms the correlation between the color transitions and electrostatic interactions of the charged species with the PDA moieties in the film, Figure 5 aims to examine the fate of the amphiphilic complexes formed between the proteins and the charged calixarene-based ligands. Figure 5A depicts adsorption isotherms of representative substances injected into the water subphase underneath a DMPC/diacetylene monolayer formed at the air/water interface. Isothermal adsorption experiments are designed to evaluate the affinity and transport of molecular species from the water subphase into amphiphilic monolayers.^{40,41}

The curves in Figure 5A depict the gradual increase of surface pressure over time as more molecules are being transferred from the aqueous phase into the DMPC/diacetylene monolayers. However, significant differences are observed among the substances examined: the calixarene ligand **2** (Figure 5A, curve a)

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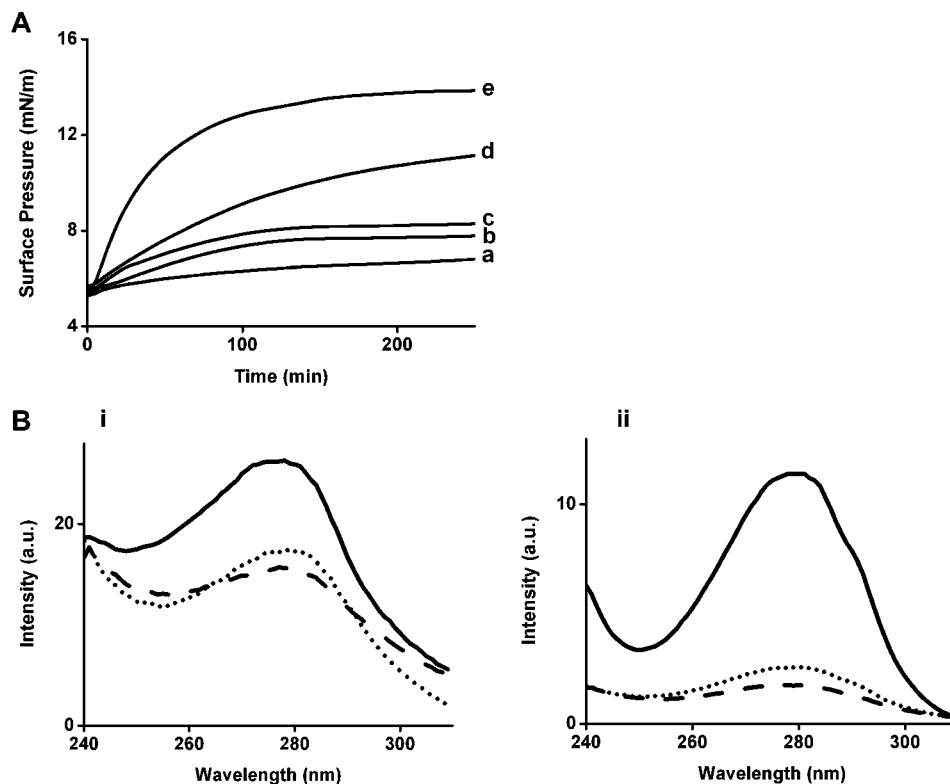


Figure 5. Incorporation of protein–ligand complexes within lipid domains. (A) Adsorption isotherms: the isotherms were recorded for DMPC/diacetylene monolayers (3:7 mole ratio) at a constant initial surface pressure (5.3 mN/m). Protein concentrations were 120 μ M, (a) ligand **2**; (b) amyloglucosidase; (c) amyloglucosidase preincubated with **2** (protein/calixarene mole ratio was 2.5:1); (d) histone; (e) histone preincubated with **2** (protein/calixarene mole ratio was 2.5:1). (B) Tryptophan fluorescence excitation (using emission at 360 nm). Spectra of glass-supported DMPC/PDA films (1:9 mole ratio) after addition of proteins and protein–ligand complexes, followed by extensive washing: (i) histone (broken line), histone preincubated with **1** (dotted line), and histone preincubated with **2** (solid line) and (ii) amyloglucosidase (broken line), amyloglucosidase preincubated with **2** (dotted line), and amyloglucosidase preincubated with **1** (solid line). Protein concentrations were 66 μ M, and the mole ratio of the protein–receptor complexes was 3:1.

and amyloglucosidase (Figure 5A, curve b) gave rise to very small increases in surface pressure, indicating a low affinity to the monolayer. A similar minor increase in surface pressure was recorded when amyloglucosidase was preincubated with **2** which also carries negative charges (Figure 5A, curve c).

Histone gave rise to a more noticeable increase in surface pressure (Figure 5A, curve d), most likely due to its electrostatic attraction to the diacetylene carboxylic acid headgroups. However, a much more pronounced rise in surface pressure was observed when histone and **2** were preincubated prior to injection into the water subphase (Figure 5A, curve e). This result can be explained by the enhanced incorporation of the amphiphilic histone–**2** complex into the phospholipid domains, thus significantly increasing the surface pressure of the DMPC/diacetylene monolayer. Previous studies have reported a similar substantial upsurge of surface pressure in lipid/diacetylene monolayer systems following incorporation of amphiphilic molecules.⁴² Significant surface pressure modulation following insertion of soluble macromolecules into amphiphilic monolayers containing calixarene-based ligands was also reported.¹⁷

Further evidence for incorporation of the amphiphilic complexes formed between charged proteins and the calixarene-based ligands into the films is provided by the tryptophan (Trp)

fluorescence excitation analysis in Figure 5B. Trp fluorescence spectroscopy affords an intrinsic probe of protein localization and its environment.⁴³ The experiments depicted in Figure 5 utilized excitation analysis rather than fluorescence emission due to the significant scattering encountered in the latter approach.

In the experiments depicted in Figure 5B, solutions containing the examined species (histone, amyloglucosidase, and these proteins preincubated with **1** or **2**) were placed upon the film surface and allowed to dry. The films were subsequently thoroughly washed with distilled water for removal of loosely attached substances, and excitation spectra were recorded (see Materials and Methods). This protocol assured observation of only the molecular species that were tightly bound or incorporated within the DMPC/PDA films.

The excitation spectra of histone, histone incubated with **1** or **2** (Figure 5B,i), and amyloglucosidase by itself and preincubated with the two ligands (Figure 5B,ii) both point to film incorporation of only the complexes formed between the opposing-charged molecules. Specifically, in the case of histone (Figure 5B,i), a significant excitation peak was apparent only in the case of preincubation of histone and the negatively charged ligand **2** (solid spectrum, Figure 5B,i). The relatively intense Trp excitation signal points to a hydrophobic environment for the residue. Much lower

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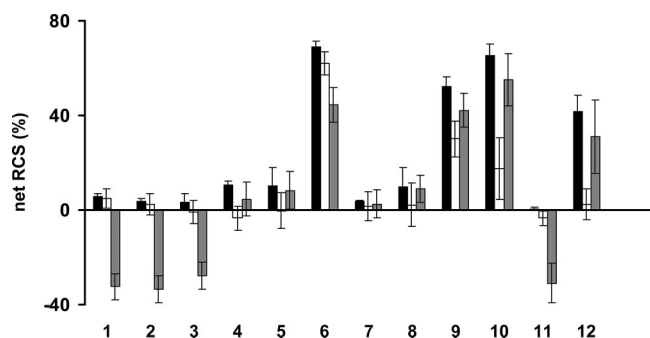


Figure 6. Quantitative colorimetric protein analysis using DMPC/PDA films: net color transitions (% net RCS, see Materials and Methods) recorded on DMPC/PDA films (1:9 mole ratio). Black columns, addition of only the protein (all concentrations were 66 μ M); white columns, protein preincubated with ligand **2** (22 μ M); gray columns, protein preincubated with ligand **1** (2.5 μ M). Proteins tested: (1) pepsin ($pI = 1.0$),⁴⁴ (2) glycoprotein acid (2.7),⁴⁵ (3) amyloglucosidase (3.6),⁴⁶ (4) trypsin inhibitor (4.6),⁴⁶ (5) β -lactoglobulin A (5.1),⁴⁶ (6) bovine serum albumin (5.3),⁴⁷ (7) chymotrypsin (8.7),⁴⁸ (8) proteinase K (8.9),⁴⁹ (9) cytochrome C (10.0–10.5),⁵⁰ (10) histone (10.2),⁵¹ (11) lysozyme (11.35),⁵² (12) polyarginine. The net RCS (%) is defined as the difference between the RCS calculated after addition of pure protein/protein–calixarene complex and the RCS induced by water/pure calixarene, respectively.

excitation signal (after film washing) was observed when histone by itself or histone preincubated with **1** were placed on the DMPC/PDA film surface (broken and dotted spectra in Figure 5B,i). Similar phenomena were observed for the acidic protein amyloglucosidase (Figure 5B,ii): intense excitation was recorded only after deposition of the amyloglucosidase–**1** complex but not after addition of the protein by itself or amyloglucosidase preincubated with **2**. Overall, the data in Figure 5 point to significant incorporation of only oppositely charged protein–ligand complexes within hydrophobic domains in the DMPC/PDA films.

Practical Application. Figure 6 presents the colorimetric application of the phospholipid/PDA films for analysis of protein surface charges. The bar diagram in Figure 6 depicts the calculated net blue–red transformations (net % RCS values) of DMPC/PDA films induced by different proteins (organized according to their isoelectric points, i.e., pI values). For each protein examined, Figure 6 presents the % RCS induced by the protein alone (subtracting the contribution of the water solution) and by the protein after preincubation with calixarenes **1** and ligand **2**, respectively (subtracting the contributions of the individual calixarene ligands to the color transformations, see Materials and Methods).

The DCA results in Figure 6 show that the DMPC/PDA films, in conjunction with the calixarene-based ligands **1** and **2**, reveal differences in abundance and organization of protein surface charge. For proteins that are highly acidic or basic, preincubation with the respective oppositely charged ligands yielded significant reduction of the colorimetric transformations of PDA, due to formation of the amphiphilic complexes as discussed above. For example, the color transitions induced by histone or cytochrome-C preincubated with the phosphonate-calixarene **2** were significantly lower than the blue–red changes induced by the free proteins. This result is ascribed to binding of the negative moieties of **2** to the positively charged surfaces of these basic proteins and subsequent formation of the amphiphilic complexes. Similarly, pepsin or amyloglucosidase preincubated with ligand **1** gave rise to significantly lower % RCS compared to **1** alone (the RCS value of **1** was 33%), again ascribed to the formation of amphiphilic complexes, with the result of reduction of the electrostatic attraction between PDA and **1**. Figure 6 features also proteins for which the colorimetric signature seems extraordinary and unrelated to the isoelectric point values.

Albumin, for example, exhibits a high RCS of around 70% even though its pI is 5.3 (i.e., mildly acidic) (protein 6 in Figure 6). Furthermore, unlike other positively charged proteins which gave rise to pronounced blue–red transitions, preincubation of BSA with **2** yielded just a small decrease of the net % RCS (Figure 6, protein 6, white column). This result might be ascribed to the high molecular weight (66–69 kDa) and corresponding large size and multidomain structure of BSA.⁵³ These characteristics mean that even though albumin is overall almost neutral, the abundance of positive domains on the protein surface results in induction of blue–red transitions when added to the DMPC/PDA film. Moreover, the organization of positive and negative domains on the protein surface⁵³ most likely interferes with binding of the phosphonate calixarene ligand **2**, resulting in the smaller attenuation of the color transformation recorded in Figure 6.

An unusual colorimetric result was also recorded for lysozyme (Figure 6, protein 11). In particular, even though lysozyme is a strongly basic protein ($pI = 11.35$), no color transformation was apparent when it was placed upon the DMPC/PDA film (Figure 6, protein 11, black column). Furthermore, the net % RCS calculated after preincubation of lysozyme with **1** was significantly negative (Figure 6, protein 11, gray bar). This surprising result indicates that lysozyme almost completely prevented the occurrence of the blue–red transformation induced by **1**. These data could be explained, however, by the prevalence of the aggregation of lysozyme on lipid surfaces.^{54,55} Indeed, the formation of a tight entwined lysozyme network at the DMPC/PDA film surface might prevent electrostatic interactions both of the protein itself or ligand **1**, giving rise to the unusual colorimetric profile observed. The seemingly anomalous results observed for albumin and lysozyme underlie the capability of the colorimetric film platform to identify proteins which properties are different than expected from their simple pI values.

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DISCUSSION

This study presents a new strategy for colorimetric analysis of protein surface charge through utilization of polydiacetylene-based films, combined with preincubation with charged calixarene-based synthetic ligands. Protein characterization was carried out via a two-stage methodology: quantification of blue–red transformations induced by a tested protein upon its interaction with the surface of a phospholipid/PDA film and subsequently evaluating the modulation of the protein-induced colorimetric transitions following preincubation of the protein examined using the synthetic ligands. The new colorimetric assay is particularly easy to implement since quantitative analysis is carried out by using conventional tabletop scanning and a simple image analysis algorithm.

The calixarene-derived ligands play two important roles in the new approach. First, their multivalency insures effective binding to charged (positive or negative) facets of the examined proteins. In addition, a direct consequence of the formation of ligand–protein complexes is the significant attenuation of the blue–red transformations in the film. This phenomenon is due to the fact that the amphiphilic complexes incorporate within the lipid domains rather than interacting electrostatically with the PDA headgroups.

Different analytical methods corroborated the proposed protein sensing model. Raman spectroscopy measurements (Figure 4) illuminated the distinct structural/chromatic transformation within the PDA backbone, which specifically corresponds to electrostatic interactions between the PDA surface and positive species, particularly basic proteins or the benzylammonium calixarene ligand. The Raman spectroscopy analysis further demonstrated that electrostatic surface interactions were reduced following ligand–protein complex formation. Adsorption isothermal analysis (Figure 5A) and tryptophan fluorescence experiments (Figure 5B) complemented the Raman spectroscopy results, pointing to the incorporation of the amphiphilic protein–ligand complexes within the phospholipid environments in the films. Overall, the experiments presented in Figures 4 and 5 support a mechanism in which modulating the electrostatic interactions between the PDA moieties and water-soluble species affects the extent of color transitions within lipid/PDA films.

The new colorimetric approach opens the way to varied applications in protein research. A straightforward utilization of

the film system in conjunction with the multivalent ligands is the evaluation of charge abundance on a protein surface. Figure 6, for example, demonstrates the significant reduction of % RCS (i.e., less pronounced blue–red transformation) for proteins displaying positive charged surfaces following their preincubation with the negatively charged phosphonate ligand **2**, while significant negative net % RCS values were obtained in the case of negative proteins preincubated with the benzylammonium calixarene **1**. Such changes in the appearance of the films constitute “visible fingerprints” for the surface charge properties of the tested proteins.

The new colorimetric approach is easy to implement for protein detection and characterization of surface charge. In particular, this method is applicable for investigating whether protein surface charge properties correlate with their *pI* or whether the macromolecules exhibit “anomalous” behavior that points to distinct structural features, such as encountered for albumin and lysozyme. Overall, the film platform can easily distinguish between very acidic, neutral, and very basic proteins. However, it might be difficult to implement the new colorimetric technique for identifying small protein charge modifications, induced, for example, in protein “charge ladders”⁵⁶ or single-residue mutants,² for which the differences in colorimetric responses between the proteins might be negligible.

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SUPPORTING INFORMATION AVAILABLE

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