Examination of Interactions of Oppositely Charged Proteins in Gels

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Understanding the interactions of proteins with one another serves as an important step for developing faster protein separation methods. To examine protein−protein interactions of oppositely charged proteins, fluorescently labeled albumin and poly-L-lysine were subjected to electrophoresis in agarose gels, in which the cationic albumin and the anionic poly-L-lysine were allowed to migrate toward each other and interact. Fluorescence microscopy was used to image fluorescently tagged proteins in the gel. The secondary structure of the proteins in solution was studied using conventional FTIR spectroscopy. Results showed that sharp interfaces were formed where FITC tagged albumin met poly-L-lysine and that the interfaces did not migrate after they had been formed. The position of the interface in the gel was found to be linearly dependent upon the relative concentration of the proteins. The formation of the interface also depended upon the fluorescent tag attached to the protein. The size of the aggregates at the interface, the fluorescence intensity modifications, and the mobility of the interface for different pore sizes of the gel were investigated. It was observed that the interface was made up of aggregates of about 1 μm in size. Using dynamic light scattering, it was observed that the size of the aggregates that formed due to interactions of oppositely charged proteins depended upon the fluorescent tags attached to the proteins. The addition of small amounts of poly-L-lysine to solutions containing FITC albumin decreased the zeta potential drastically. For this, we propose a model suggesting that adding small amounts of poly-L-lysine to solutions containing FITC −albumin favors the formation of macromolecular complexes having FITC albumin molecules on its surface. Although oppositely charged FITC tagged poly-L-lysine and FITC tagged albumin influence each other's migration velocities by forming aggregates, there were no observable secondary structural modifications when the proteins were mixed in solution.

Introduction

Proteomics deals with both qualitative identification and quantitative measurements of proteins using a combination of electrophoresis, mass spectrometry, and bioinformatics.[1−4] The need for the precise identification of unknown proteins has led to several innovations in separation techniques that include two-dimensional polyacrylamide gel electrophoresis (2-D page), capillary zone electrophoresis (CZE), capillary isoelectric focusing (ICEF), and sieving SDS capillary electrophoresis (sieving SDS-CE).[5−8] Proteins show a great diversity in their charge content and in their structures. Understanding the interactions of proteins with one another serves as an important step for developing faster protein separation methods. Studies relating to protein−protein interactions can also lead to a better understanding of the cellular mechanism that involves protein−protein interactions. Unlike the behavior of DNA during electrophoreses, which are almost entirely a function of size, protein electrophoreses, on the other hand, because of the highly variable 3-D properties of proteins and interprotein interactions, are much more complex.[9] In this article, we investigate the interaction of oppositely charged proteins undergoing electrophoresis in agarose gels, in which the cationic protein and the anionic protein were

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allowed to migrate toward each other and interact. Fluorescently labeled bovine serum albumin and casein were chosen as cationic proteins, and poly-L-lysine was chosen as the anionic protein. Albumin makes up 55−62% of blood proteins and is one of the few carbohydrate-free proteins in plasma. The main function of albumin is the regulation of the colloidal osmotic pressure of blood. It is a globular water soluble protein with 55% α-helical and 45% disordered structure[10−13] and has an isoelectric point of 4.7.[13] Casein micelles are colloidal complexes of protein and salts that function biologically to transport efficiently and deliver protein, calcium, and phosphorus to the neonate.[14−19] Casein assumes submicelles of αs1, αs1, β, and κ forms. Poly-L-lysine is a positively charged synthetic amino acid polymer that is used for promoting cell adhesion to solid substrates. Poly-L-lysine enhances the electrostatic interaction between negatively charged ions of the cell membrane and the culture surface,[20−23] and it forms complexes with DNA and has applications as a gene delivery tool.[24−26] The chemical structure and the conformation of poly-L-lysine in solution have been well-characterized.[27−30] Also, poly-L-lysine has been found to form large micrometer sized aggregates with gold nanoparticles.[31] Therefore, in addition to investigating the interactions of oppositely charged proteins in gels, we also investigated the zeta potential modifications and the size changes associated with protein−protein interactions in solutions using zeta potential measurement techniques and dynamic light scattering techniques, respectively.

Experimental Procedures

Materials and Methods. Proteins. The fluorescently labeled proteins FITC (fluorescein isothiocyanate) labeled albumin (mol wt 66 kDa) with 7−10 mol of FITC (mol wt 389.4) per mol of albumin (FITC albumin); FITC labeled casein (mol wt 24 kDa) with 1.1 mol of FITC per mol of casein (FITC casein); FITC labeled poly-L-lysine (mol wt 67.6 kDa) with 2.3 mol of FITC per mol of poly-L-lysine (FITC poly-L-lysine); TRITC (tetramethylrhodamine isothiocyanate) labeled albumin (mol wt 66 kDa) with 1 mol of TRITC (mol wt 479 kDa) per mol of albumin (TRITC albumin); BSA (bovine serum albumin) of mol wt 66 kDa; and poly-L-lysine hydrochloride (mol wt > 30 000) were purchased from Sigma. The fluorescent tags were coupled to the proteins through the ϵ amino group of lysines of the proteins. The procedure for reaction of isothiocyanate functionality with amino group lysines is the same for labeling both FITC albumin and TRITC albumin. FITC has an absorption maximum at 495 nm and emission maximum at 525 nm. TRITC has an absorption maximum at 547 nm and emission maximum at 572 nm.

Electrophoresis. NuSieve GTG Agarose (a low melting temperature agarose) was purchased from Cambrex Bio Science Rockland, Inc. for making the gel. The electrophoresis experiments were carried out in a 100 mM potassium phosphate solution at pH 7.0. Albumin and casein have isoelectric point values of ∼4.5; hence, they are negatively charged (cationic) in a buffer of pH 7.0. Poly-L-lysine has a PI value of ∼9.2. Hence, poly-L-lysine has a positive charge (anionic) in potassium buffer of pH 7.0. For the electrophoresis experiments, the wells were loaded with a maximum of 10 μL of the protein solution along with 20% by volume of glycerol. The migrating proteins were observed at various times during electrophoresis using a Kodak Digital Sciences Imaging Station 440.

Characterization. Confocal Laser Scanning Microscopy. Confocal images were captured with a Leica TCS SP2 confocal microscope equipped with 10× and 40× objectives. The laser excitation wavelength of 488 nm was chosen for FITC ($\lambda_{ex} = 494.5$ nm and $\lambda_{em} = 519$ nm). Samples were mounted on conventional glass slides.

Measurement of the Size of the Aggregates in the Gel. For studying the size of the aggregates of proteins trapped in a gel, 10 μm thin slices of the gel were cut using a cryomicrotome. The thin slice was collected on a glass cover slip by gently placing the cover slip on it. The interface region was imaged using confocal microscopy. The size of the aggregates was analyzed by performing line scans using confocal microscopy.

Measurement of the Size of Aggregates in Solution. For studying the size distribution of proteins in solution, a 100 mM potassium phosphate solution containing proteins of concentration 1 mg/mL was placed in 4 mL polystyrene cuvettes. The samples were gently mixed with the help of the pipette. The size distribution of the protein aggregates in a solution was studied using a Brookhaven Zeta Plus dynamic light scattering (DLS) system equipped with a BI-9000AT digital autocorrelator at a 659 nm wavelength. All studies were done at a 90° scattering angle and were temperature controlled at 25 °C. The number of runs for each sample was set as 10, and the run duration was set as 1 min using the software package 9KDLSW. The correlation function was interpreted using the algorithms NNLS, CONTIN, and EXPSAM. Particle size distributions that were common with at least two of three algorithm programs were considered for data interpretation. Only those particle distributions that followed a trend were considered for data interpretation. The size distributions were also compared to the effective diameter of the particles and the particle count rate to check for correlations among the factors.

Zeta Potential Analysis of Proteins in Solution. Zeta potentials were calculated using phase analysis light scattering (PALS), a variation of electrophoretic dynamic light scattering (DLS).[38] The Smoluchowski limit (i.e., $\kappa a \gg 1$), where κ is the Debye−Huckel parameter and a is the particle radius in Henry's equation,[33] was applied to the calculation of the zeta potential from electrophoretic mobility measurements. A dip-in electrode system with 4 mL polystyrene cuvettes was used.[34] The electric field applied was ∼5.1 V cm^{-1}. The background electrolyte was chosen as 25 mM potassium phosphate since the current observed when 100 mM potassium phosphate was used as background electrolyte was very large (∼40 000 μS). All studies were temperature controlled at 25 °C. The number of runs for each sample was set as 10, and the number of cycles for each run was set as 10. Out of the 10 readings, two of them having the highest values and two of them having the lowest values were not considered for data interpretation to avoid possible errors introduced due to temperature fluctuations when the DLS measurements were begun. The other six intermediate values were chosen for data interpretation.

Secondary Structure of Proteins in Solution. For studying the secondary structure of the proteins in solution, 20 μL of solution

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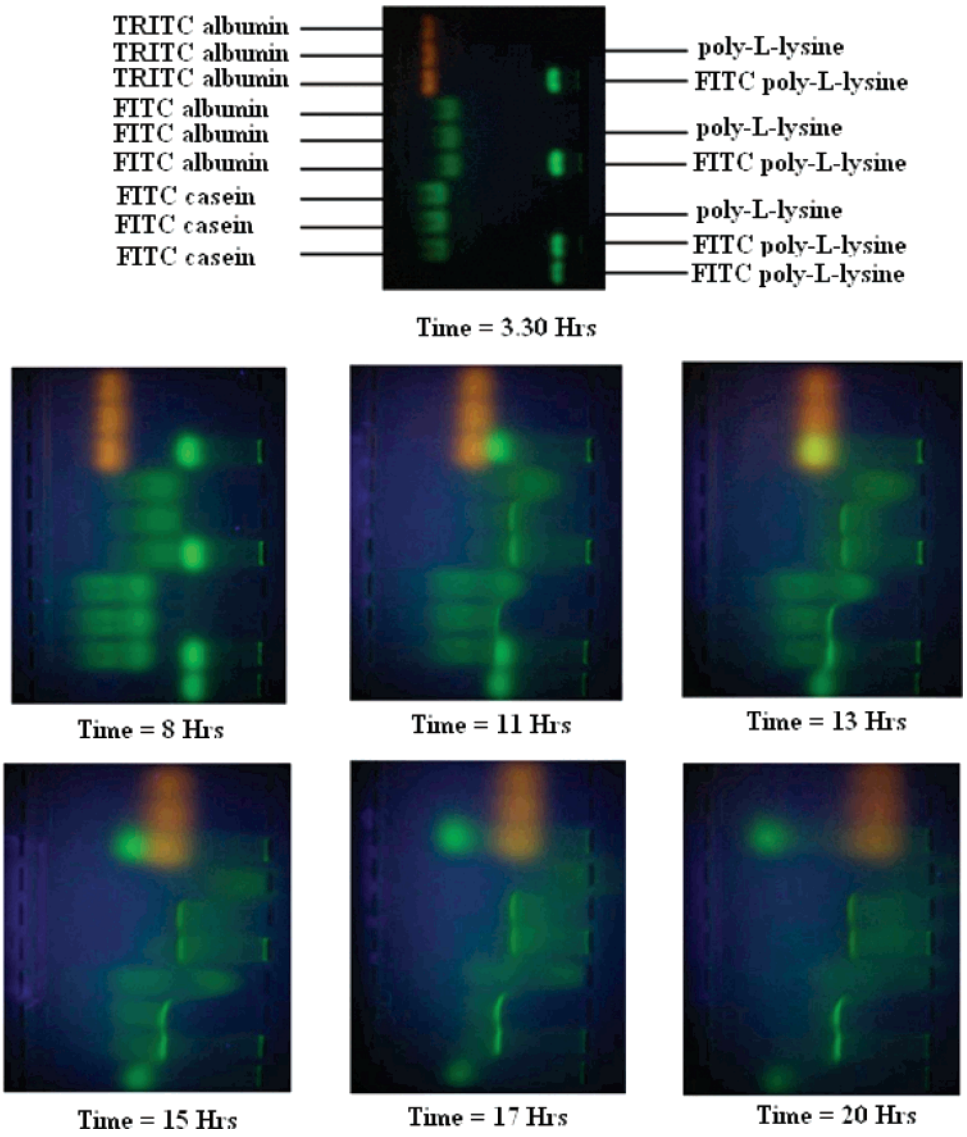

Figure 1. Formation of various protein−protein interfaces as a function of time in 2% agarose gel.

containing 10 mg of protein per 1 mL of the solution was placed between two calcium fluoride disks separated by a 25 μm Teflon spacer. A Thermo Nicolet Magna 560 FTIR spectrometer equipped with a KBr beamsplitter and DTGS detector was used for FTIR analysis of the protein structure in solution. The spectral resolution was 4 cm^{-1}, and 128 scans per point were collected. The background was collected by using a 100 mM potassium phosphate solution at pH 7.0.

Results and Discussion

Proteins in Gel. Electrophoreitc Mobility of Proteins in Agarose Gel. The electrophoresis of proteins was done in 1, 2, and 3% (w/v) agarose gels with 100 mM potassium phosphate as the electrolytic buffer. It was found that TRITC albumin migrated at a slower rate as compared to FITC albumin. With time, FITC albumin and FITC casein spread into broad bands, while TRITC albumin and FITC poly-L-lysine remained as narrow bands. For a 2% gel run in 100 mM potassium phosphate at pH 7.0, the mobilities of FITC albumin, TRITC albumin, FITC casein, and FITC poly-L-lysine are 12×10^{-5}, 7.5×10^{-5}, 9.75×10^{-5}, and 7×10^{-5} cm^2 V^{-1} s^{-1}, respectively. The mobility of the proteins was found to be the same in 1 and 2% agarose gels. For the 3% agarose gel, the mobilities of FITC albumin, TRITC albumin, FITC casein, and FITC poly-L-lysine are 9.12×10^{-5}, 5.47×10^{-5}, 7.9×10^{-5}, and 5.47×10^{-5} cm^2 V^{-1} s^{-1}, respectively. Hence, the pore size for 2 and 1% agarose gels did not change the mobility of the proteins. Since FITC albumin had a higher mobility than TRITC albumin and since the amount of FITC conjugated to albumin is higher than the amount of TRITC conjugated to albumin, we assume that the FITC molecule had changed the net charge of albumin to a greater extent than in the case of TRITC.

Formation of Interfaces in Agarose Gel. To observe the interaction of proteins in gels, FITC albumin, TRITC albumin, untagged BSA, and FITC casein were loaded in different wells in the cathode end of the electrophoretic cell, while FITC poly-L-lysine was placed in the anode end. An electric field of 1 V cm^{-1} was applied. It was observed that sharp interfaces were formed where FITC albumin and FITC casein met poly-L-lysine or FITC poly-L-lysine in the gel. However, no apparent interface was formed where TRITC albumin or untagged BSA met poly-L-lysine or FITC poly-L-lysine. These results are shown in Figure 1. To determine if the applied electric field was responsible for the disruption of the formation of the interfaces when TRITC albumin or BSA met poly-L-lysine during electrophoresis, the field was switched off when the proteins met and restarted after 12 h. It was still observed that TRITC albumin or BSA did not

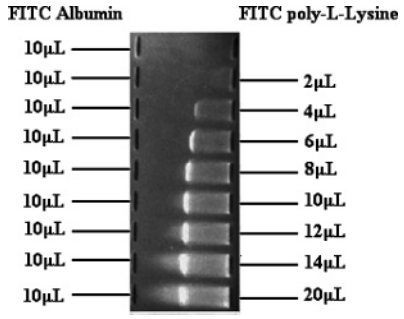

Figure 2. Position of FITC albumin: FITC poly-L-lysine interface as a function of FITC poly-L-lysine concentration.

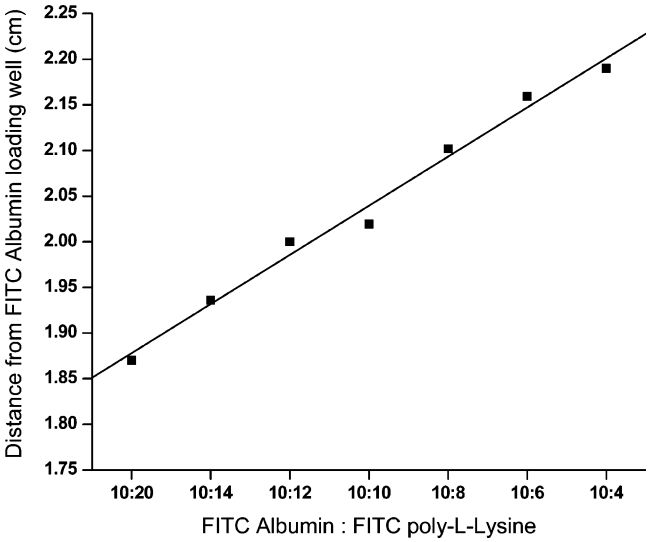

Figure 3. Linear dependence of the distance (in cm) of the interfaces from wells in which FITC albumin was initially loaded (Figure 2) as a function of the relative concentrations of the proteins.

form interfaces with poly-L-lysine while FITC albumin did. Hence, the applied electric field did not influence the formation or subsequent disruption of the interfaces. This suggests that the formation of the interface is an evidence of some sort of chemical bonding occurring when oppositely charged poly-L-lysine and FITC albumin interact.

Confocal microscopy can be used to focus on any particular plane in a sample. To check if the interface was uniform throughout the height of the gel, confocal microscopy was used to image the interface at various heights, starting from the bottom surface of the gel to the top surface of the gel. It was found that the interface had the same profile at all heights. It was also found that the interface had maximum fluorescence intensity when compared to the other regions of the gel. The positions of the interface for varying concentrations of the proteins were close. The relative position of the interfaces did not change when the pH of the potassium phosphate buffer was varied between 6, 7, and 8, although the mobility of the individual proteins varied with the pH of the buffer. Interfaces formed by varying the concentrations of FITC albumin and FITC poly-L-lysine are shown in Figure 2. The linear dependence of the distance of the interfaces from wells in which FITC albumin was initially loaded in Figure 2 (in cm) as a function of the concentrations of the proteins is shown in Figure 3. This suggests that the loading volumes do play a significant role in the formation of the interfaces. Figure 4 shows the position of the bands of FITC albumin and FITC poly-L-lysine before and after they meet as a function of the time for various concentrations of the proteins, when a field of 1 V cm^{-1} is applied. Figure 4 clearly shows that

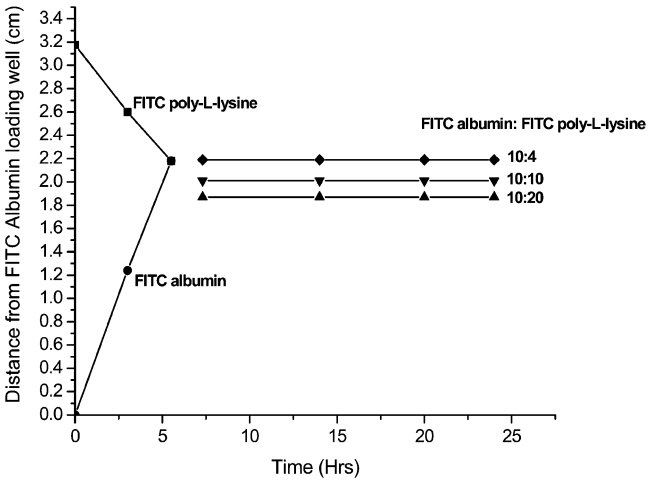

Figure 4. Position of FITC albumin and FITC poly-L-lysine before and after they meet as a function of time for various concentrations.

the bands are immobilized when they meet. It also shows that the position of the interface depends upon the concentration of the proteins. For 2 and 3% agarose gels, the interfaces were formed when the ratio of poly-L-lysine to FITC albumin exceeded 2:5. This suggests that the protein mixtures have to reach a certain stoichiometry to form interfaces. It was observed that while the interface formed, some proteins also pass the interface and proceed toward their respective electrodes. The proteins might neutralize one another and therefore remain immobile or form complexes that become stuck in the gel due to the physical constraint imposed by the pore size of the gel.

Measurement of the Size of the Aggregates in the Interface Region of the Gel. Observing the interior regions of the interface in bulk gels using confocal microscopy or fluorescence microscopy is not possible due to the overlap of fluorescence emission from neighboring regions that cannot be resolved using fluorescence microscopy. Therefore, to image the interior regions of the interface, 10 μm thin sections of the region containing the interface formed by interaction of FITC albumin and poly-L-lysine in 2% agarose gel were cut using a cryomicrotome. The 10 μm thin section was collected on a glass slide. Exposure to room temperature conditions made the gel dissociate due to evaporation of water, leaving the proteins trapped in the interface region of the gel. The interface region was imaged using a confocal microscope (Figure 5), and the size distribution of the aggregates at the interface as observed using confocal microscopy is shown in Figure 6. It was observed that the interface was made up of aggregates of size $\sim$1.3 μm.

Measurement of the Size of Aggregates in Solution using Dynamic Light Scattering. DLS measurements were made on samples containing proteins and their mixtures in 100 mM potassium phosphate solution. It was observed that tagged and untagged albumin, casein, and poly-L-lysine had sizes $<$10 nm. When poly-L-lysine was added to FITC albumin, the size of the aggregates increased more rapidly with respect to the concentration of poly-L-lysine than in the case of poly-L-lysine added to BSA or TRITC albumin. The size of the aggregates for equal molar mixtures of poly-L-lysine to FITC albumin was as high as $\sim$1300 nm, while that for albumin and TRITC albumin was $\sim$500 and $\sim$50 nm, respectively. The size distributions of the aggregates for mixtures of poly-L-lysine and FITC albumin, poly-L-lysine and BSA, and poly-L-lysine and TRITC albumin are shown in Figure 7. The size of the poly-L-lysine $-$FITC albumin aggregates measured by DLS is in good agreement with the size of the poly-L-lysine $-$FITC albumin aggregates in the interface

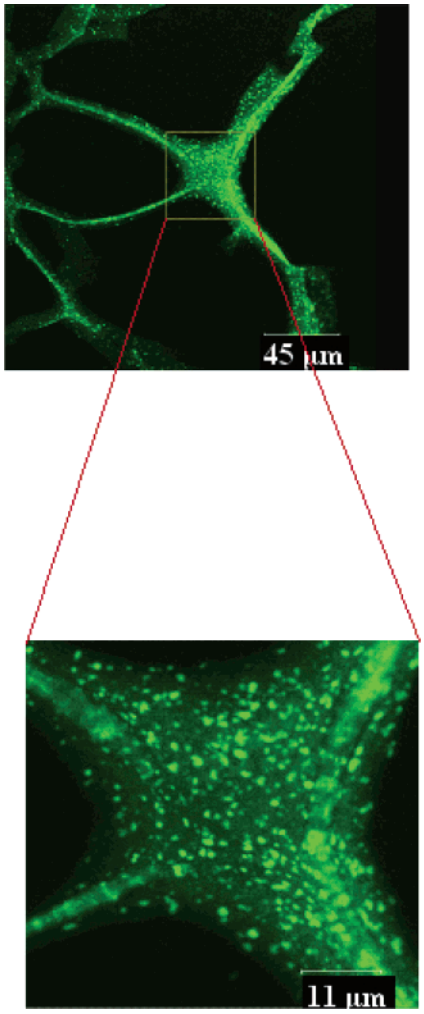

Figure 5. Confocal image of the aggregates in the interface formed by FITC albumin and poly-L-lysine in 2% agarose gel.

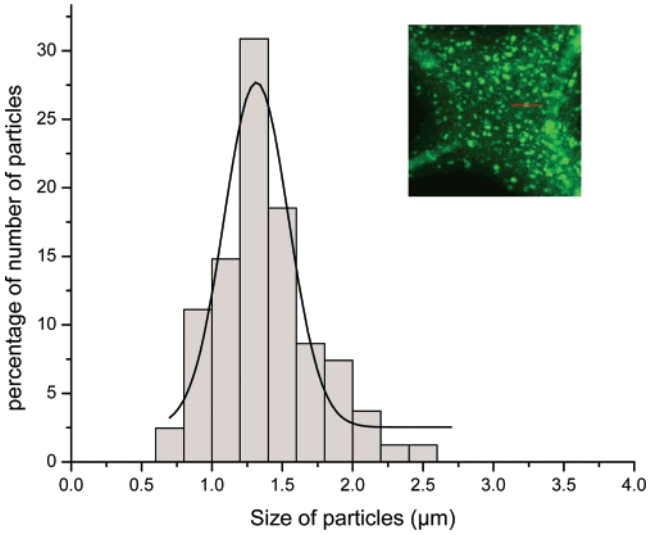

Figure 6. Size distribution of the aggregates at the interface formed by FITC albumin and poly-L-lysine found by performing a line scan (red line in the confocal image) on the aggregates using a confocal microscope.

region of a gel measured by confocal microscopy (Figure 6). The size distribution for mixtures of poly-L-lysine and FITC casein is similar to that of mixtures of poly-L-lysine and FITC albumin.

Zeta Potential Measurements in Solution. When zeta potential measurements were made using a 100 mM potassium phosphate solution as the background electrolyte, it was observed that the current drawn was very large (~40 000 μS). Therefore, the zeta potential of the proteins was measured using a 25 mM potassium phosphate solution at pH 7.0. The zeta potentials of tagged and untagged proteins were the same. The zeta potentials of albumin, casein, and poly-L-lysine were found to be −15, −22, and 15 mV, respectively. Two phenomena were observed while increasing the concentration of poly-L-lysine in solutions containing tagged and untagged albumin or casein: one for lower concentrations of poly-L-lysine and one for higher concentrations.

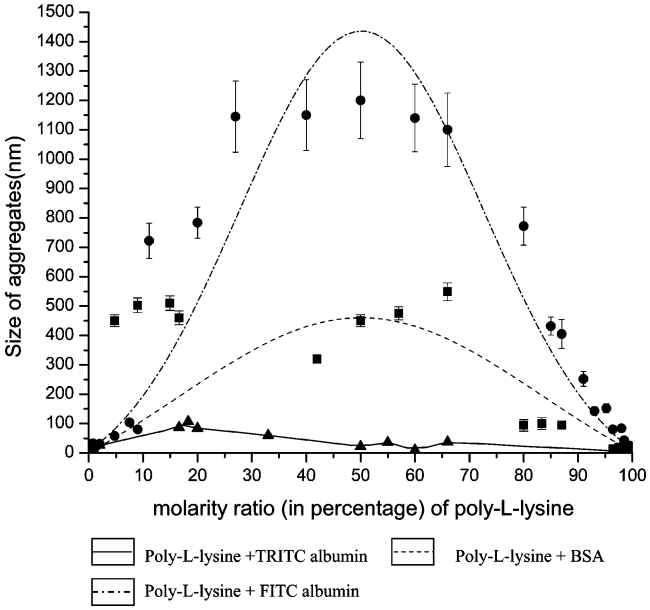

Figure 7. Size of aggregates of a mixture of proteins in a 100 mM potassium phosphate solution of pH 7.0.

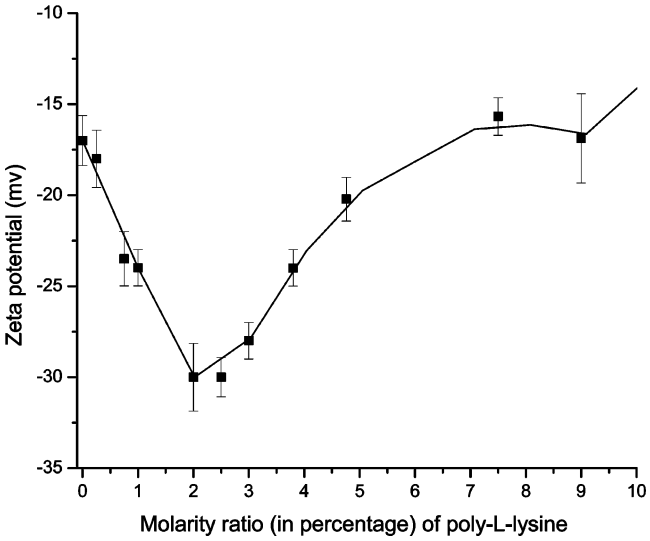

Figure 8. Zeta potential for mixture of poly-L-lysine and FITC albumin in 100 mM potassium phosphate.

For Lower Concentrations of Poly-L-lysine. When poly-L-lysine was added to FITC albumin, it was observed that the zeta potential value initially decreased until the molarity percentage ratio of poly-L-lysine in the solution became 2% and then increased with an increasing concentration of poly-L-lysine (Figure 8). There are two possible explanations for this phenomenon.

Model 1. The zeta potential value is measured using the mobility of the particle. The mobility of a particle depends upon the applied electric field, the electro-osmotic flow due to the

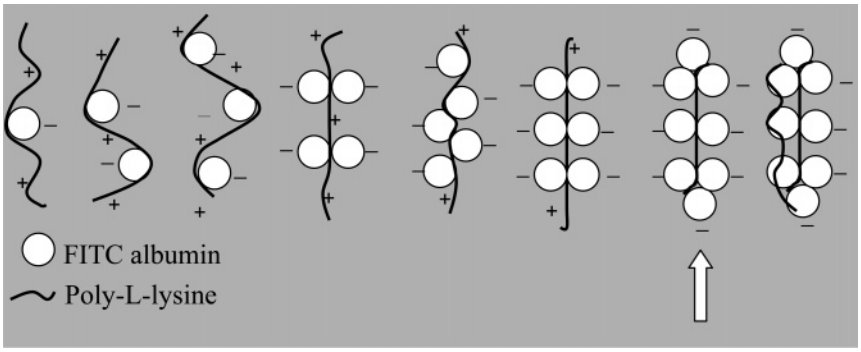

Figure 9. Schematic representation of model 2 showing the formation of a single macromolecular complex having a negative surface charge. At low concentrations, poly-L-lysine serves as a scaffold to which negatively charged FITC albumin molecules attach. The surface charge of the macromolecular complex becomes more negative as more FITC albumin molecules become attached to a poly-L-lysine molecule until the FITC molecules completely cover the poly-L-lysine molecule resulting in a negative peak of the zeta potential (indicated by the white arrow in the figure). Increasing the concentration of poly-L-lysine (last picture in the figure) decreases the negative charge of the macromolecular complex due to charge screening.

Table 1. Size Distribution of Protein Aggregates at Low Concentrations (≤5%) of Poly-L-lysine

molarity ratio (in %) of poly-L-lysine	BSA	TRITC albumin	FITC albumin	FITC casein
0	∼5 nm	∼5 nm	∼5 nm	∼10 nm
0.25	5.2 ± 0.74	9 ± 2.3	15 ± 2.4	14 ± 0.9
0.5	7 ± 1.4	15 ± 3.1	20 ± 2.3	1 4 ± 09
0.75	7.5 ± 1.2	20 ± 2.2	23 ± 2.3	18 ± 1.4
1	18 ± 1.4	23 ± 2.1	25 ± 2	24 ± 2.6
2	33 ± 2.5	26 ± 1.9	32 ± 3	34 ± 3.1
5	55 ± 3.2	50 ± 4.3	58 ± 8	60 ± 5.8

buffer ions, and the electrostatic attraction and repulsion due to unlike and like charged neighboring particles. In the absence of the positively charged poly-L-lysine, the zeta potential value of FITC albumin is −15 mV. The addition of slight amounts of poly-L-lysine (∼2%) can screen the electrostatic repulsion forces between FITC albumin molecules, thereby increasing the mobility of the particles. Therefore, the zeta potential value can become as low as −30 mV when a small amount of poly-L-lysine is added to albumin.

Model 2. The sizes of the aggregates for mixtures of proteins for low concentrations of poly-L-lysine as measured by DLS are shown in Table 1. From the DLS measurements, it was observed that at low concentrations of poly-L-lysine (∼2%), the size of the aggregates was ∼30−40 nm for mixtures of poly-L-lysine and FITC albumin. Since the size of individual molecules of both tagged and untagged albumin is ∼5 nm and that of poly-L-lysine is ∼15 nm, this suggests that −four to eight molecules of albumin might bind to the poly-L-lysine molecule in such a way that both sides of poly-L-lysine are attached to the negatively charged FITC albumin molecule. The positive charge of the albumin will therefore be screened by the FITC albumin molecule. This intact group of molecules can move as a single macromolecular complex. The surface charge of this single macromolecular complex is determined by the albumin molecules as they are in its surface, and therefore, the surface charge becomes ∼2−3 times as large as that of FITC albumin (Figure 9). This, in turn, can result in mobilities approximately twice as large as that of a single molecule of albumin. Further addition of poly-L-lysine to the solution will result in the reduction of surface charges of the macromolecular complex since attachment of a new poly-L-lysine molecule results in partial coverage (as the size of the single macromolecule is ∼30−50 nm, while that of poly-L-lysine is only ∼15 nm). Although the fraction of these macromolecular complexes might be less than that of the number of free unbound albumin molecules in solution (due to low concentrations of poly-L-lysine), the size may be significant enough for the laser beam in the zeta potential experiments to detect its migration in preference to that of the single molecules of albumin due to their smaller sizes. This is confirmed by observing the steep increase in the particle count rate made during zeta potential measurements. The particle count rates observed when poly-L-lysine was added to negatively charged proteins made during zeta potential measurements are presented in Table

Table 2. Particle Count Rate in Kilo Counts Per Second (KCPS) Observed during Zeta Potential Measurements

molarity ratio (in %) of poly-L-lysine	BSA	TRITC albumin	FITC albumin	FITC casein
0	∼200	∼800	∼200	∼1200
0.25	269	1345	1706	1370
0.5	1719	1854	1812	1465
0.75	1725	1577	1485	1960
1	1735	1852	1315	1655
2	1636	1854	1652	1576
4.75	1647	1939	1916	1536
7.5	1332	1783	1709	1517
9	1397	1847	1749	1732
11.1	1618	1677	1903	1478
13	1576	1760	1665	1060
14.9	1174	1833	1857	1782
16.6	1636	1746	1138	1432
20	1472	1814	910	1235

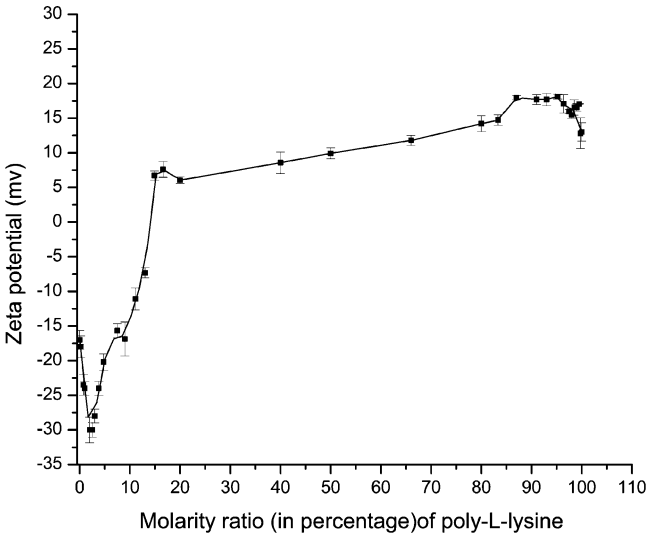

Figure 10. Zeta potential of a mixture of FITC albumin and poly-L-lysine in 100 mM potassium phosphate solution.

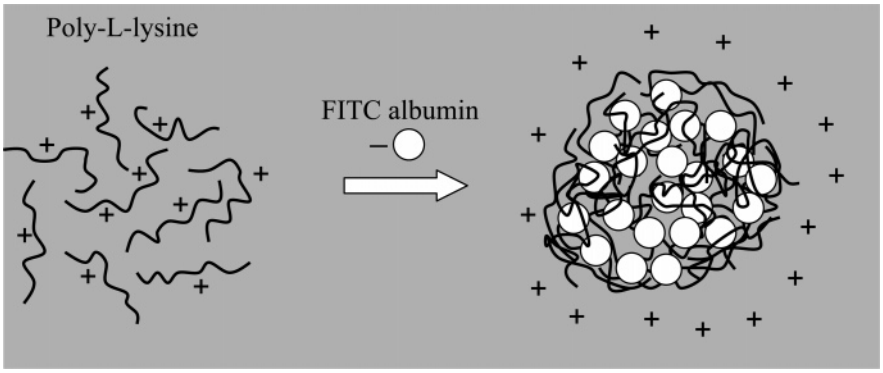

Figure 11. Formation of positively charged complexes when poly-L-lysine is added to FITC albumin for percentage molarity ratio of poly-L-lysine greater than ~20%.

Table 3. Zeta Potential (mV) of Individual Proteins and Their Mixtures

molarity ratio (in %) of poly-L-lysine	BSA	TRITC albumin	FITC albumin	FITC casein
0	−15 ± 1.8	−12 ± 1	−17 ± 1.37	−24 ± 0.72
0.25	−15.1 ± 2.1	−11.1 ± 1.2	−18 ± 1.57	24.5 ± 0.84
0.5	−15.3 ± 4.3	−9.44 ± 0.87	−24.8 ± 1.9	−25 ± 1
0.75	−15.8 ± 2.16	−9.74 ± 0.9	−23.49 ± 1.4	−25.6 ± 0.75
1	−15.9 ± 1.23	−9.99 ± 0.75	−23. ± 1	−23.75 ± 1
2	−12.94 ± 1	−8.87 ± 0.58	−30 ± 1.86	−21.8 ± 3.43
4.75	−8.6 ± 0.6	−1.84 ± 2.17	−20.2 ± 1.2	+6.25 ± 0.56
7.5	−5.94 ± 0.95	+3.85 ± 0.2	−15.7 ± 1	+8.4 ± 0.75
9	−5.85 ± 0.96	+3.77 ± 0.61	−16.88 ± 2.45	+8.45 ± 0.9
11.1	+0.7 ± 2.27	+5 ± 0.67	−11.1 ± 1.5	+8.0 ± 1
13	+3.94 ± 0.66	+7.98 ± 0.72	−7.33 ± 0.73	+8.6 ± 1.1
14.9	+7.49 ± 1.27	+7.55 ± 0.4	+6.72 ± 0.68	+8.75 ± 0.9
16.6	+7.91 ± 0.75	+7.85 ± 0.54	+7.61 ± 1.4	+8.8 ± 1.2
20	+7 ± 0.91	+7.02 ± 0.44	+8.5 ± 1.53	+8.9 ± 0.8

2. However, this effect of initial decrease and then increase in the zeta potential value was more prominent when poly-L-lysine was added to FITC albumin than when added to FITC casein or BSA. When poly-L-lysine was added to TRITC albumin, this effect was not observed.

For Higher Concentrations of Poly-L-lysine. As the molarity of poly-L-lysine in solutions containing tagged and untagged albumin or casein increased, the zeta potential increased from negative to positive values. The change in the sign of the zeta potential when the concentration of poly-L-lysine added to FITC albumin is increased is shown in Figure 10. The variation in the zeta potential value with increasing amounts of poly-L-lysine in 25 mM potassium phosphate solution for different proteins is presented in Table 3. The zeta potential became zero for albumin and casein when the percentages molarity of poly-L-lysine added to them were 11 and 4.2%, respectively. This could be because of the size difference between albumin molecule and casein molecule since the molecular weight of albumin (mass 66 kDa) is 2.75 times that of the casein molecule (mass 24 kDa). Poly-L-lysine has a random coil as its secondary structure, while albumin is a globular protein. Some or all parts of poly-L-lysine might wrap around the albumin molecule while exposing its unattached side to the electrolyte or to another albumin molecule. When the number of poly-L-lysine molecules becomes sufficiently large, upon application of an electric field, the side of the poly-L-lysine molecule that faces the electrolytic buffer can give an overall positive value for the zeta potential since the negatively charged surface of the albumin molecules has been screened by the poly-L-lysine molecules that encapsulate them (Figure 11). From DLS experiments, the typical size of the aggregates for mixtures of poly-L-lysine and FITC albumin for which the zeta potential value is zero is ~300−500 nm. The size of the aggregates observed in the gel (Figure 5) is ~1300 nm. This suggests that the aggregates trapped in the gel are positively charged.

Effect of Addition of NaCl on Interfaces. The interface that was formed when the proteins met became sharper with time during electrophoresis. Their positions after they had been formed remained fixed and did not change even after subjecting the proteins to electrophoresis for more than 24 h. When the polarity of electrophoresis was reversed, the band did not separate, and it remained intact. To find the effect of the addition of NaCl upon the size of the protein aggregates in solution, dynamic light scattering measurements were made as greater amounts of 5 M

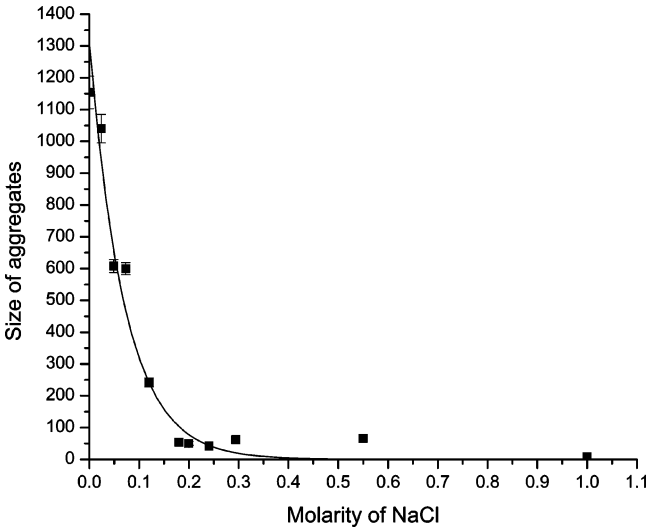

Figure 12. Size of aggregates in 100 mM potassium phosphate solution containing equal molar concentrations of FITC albumin and poly-L-lysine as a function of concentration of NaCl.

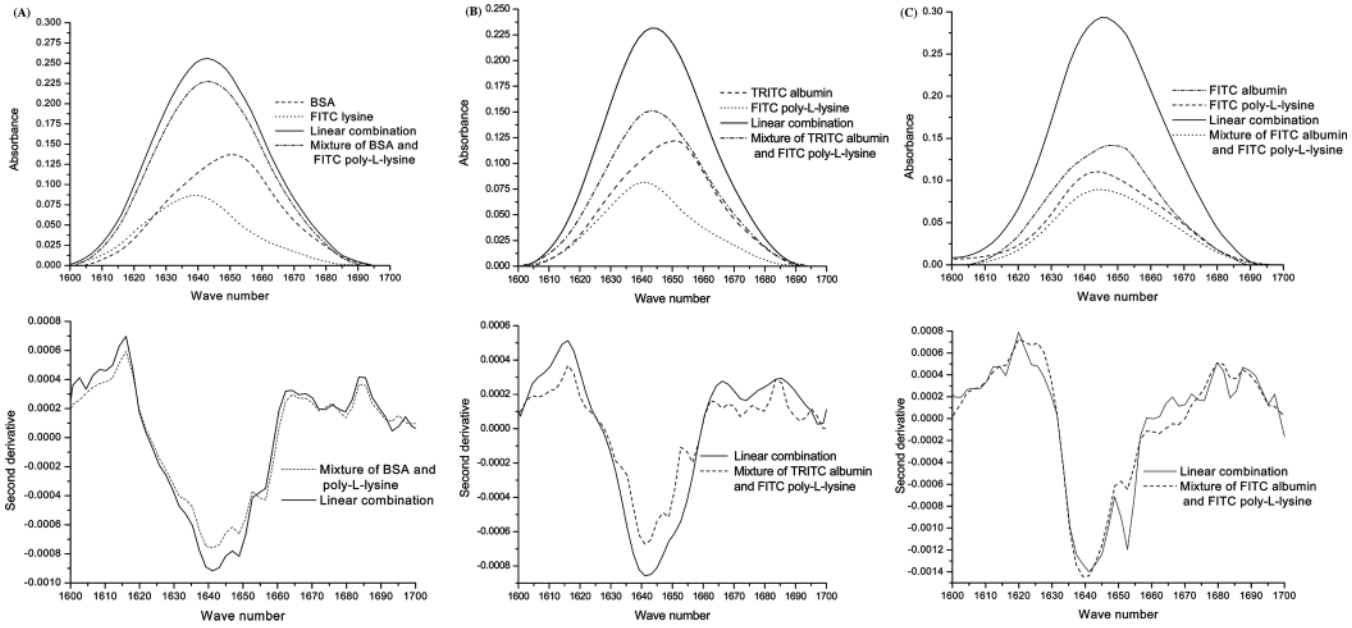

Figure 13. (A) Comparison of FTIR spectra of equal mixtures BSA and poly-L-lysine with linear combinations of spectra from both proteins individually. Both spectra are similar, suggesting that there are no secondary structure modifications when BSA and poly-L-lysine are mixed. (B) Comparison of FTIR spectra of equal mixtures TRITC albumin and FITC poly-L-lysine with linear combinations of spectra from both proteins individually. Both spectra are similar, suggesting that there are no secondary structure modifications when TRITC albumin and FITC poly-L-lysine are mixed. (C) Comparison of FTIR spectra of equal mixtures FITC albumin and FITC poly-L-lysine with linear combinations of spectra from both proteins individually. Both spectra are similar, suggesting that there are no secondary structure modifications when FITC albumin and FITC poly-L-lysine are mixed.

NaCl were added to the 100 mM potassium phosphate solution containing equal molarity concentrations of FITC albumin and poly-L-lysine. The size dependence of the aggregates upon the concentration of NaCl in 100 mM potassium phosphate solution is shown in Figure 12. It was observed that the size of the aggregates decreased exponentially with increasing concentrations of NaCl and that the size of the aggregates was less than 10 nm for 1 M NaCl in potassium phosphate solution. Hence, the addition of NaCl dissociates the aggregates, and for 1 M NaCl in a potassium phosphate solution, the proteins were completely separated. The formation of the interface in the gel appears to be due to strong electrostatic interactions of the proteins. To confirm this, equal mixtures of FITC albumin and FITC poly-L-lysine were first mixed, then loaded in the gel, and when subjected to electrophoresis, the proteins did not migrate into the gel, possibly due to the size of the protein aggregates being larger than that of the pore size of the gel. When 1 M NaCl was included in the mixture of FITC albumin and FITC poly-L-lysine and then loaded in the gel for electrophoresis, the proteins did not appear to interact; instead the proteins separated and moved toward their respective electrodes. On the other hand, when the parts of the gel containing the interface were soaked in 1 M NaCl for 1 h and then subjected to electrophoresis, the interface remained intact, although with time the intensity of the fluorescence gradually diminished, possibly due to the dissolution of the gel in a high NaCl concentration. This suggests that the protein aggregates are strongly packed at the interface. These findings suggest that the interaction between FITC albumin and FITC poly-L-lysine is electrostatic and can be prevented but, once formed, not disrupted by high salt concentrations.

Secondary Structure of Proteins in Solution. The secondary structure was studied with FTIR by examining the second derivatives of the absorbance spectra in the amide I spectral region (1600−1700 cm^{-1}). To check if the secondary structure was modified by tagging the proteins with fluorescent tags, the secondary structure of BSA, FITC albumin, TRITC albumin, and FITC poly-L-lysine were studied in D_2O/100 mM potassium phosphate solution. It was found that the secondary structure of the non-tagged proteins was the same as that of the tagged proteins (data not shown) in the amide I band region. For albumin, the second derivative peak was at 1652 cm^{-1}. This corresponds to an α-helical secondary structure.[35] FITC casein and FITC poly-L-lysine had their second derivative peaks at 1641 cm^{-1}. This corresponds to a random coil structure.[35] Albumin and poly-L-lysine were mixed at equal volumes and concentrations, and another FTIR spectrum was collected. The resulting spectrum was identical to a linear combination of the individual albumin and poly-L-lysine spectra. This result indicates that the secondary structures associated with the functional groups in the amide I band region of these two proteins do not show any observable change in solution when they interact in solution. The same observations were made when the TRITC albumin and FITC albumin were mixed with poly-L-lysine. These results are shown in Figure 13A−C. However, since FTIR is a bulk technique, it is possible that some near-surface changes might occur when oppositely charged proteins interact.

Possible Explanation for Surface Alteration of Albumin by FITC. FITC tagged albumin has a higher electrophoretic mobility than TRITC tagged albumin in gels. This suggests that FITC tagged albumin has more surface charge than TRITC tagged albumin or untagged BSA and that the molecule forms an interface with a positively charged poly-L-lysine molecule. Asparatic acid (D) and glutamic acid (E) are amino acids with strong negative charge that are hydrophilic and are usually located in the outer surface of the proteins. For FITC tagged albumin, the FITC molecule is tagged to the ϵ amino groups of the lysine (K) of albumin. The 3-D image of the albumin molecule with the acidic residues (asparatic acid and glutamic acid) and basic residues (arginine and lysine) is shown in Figure 14. Using a Cn 3-D tool for visualization of protein structures, it was found that out of

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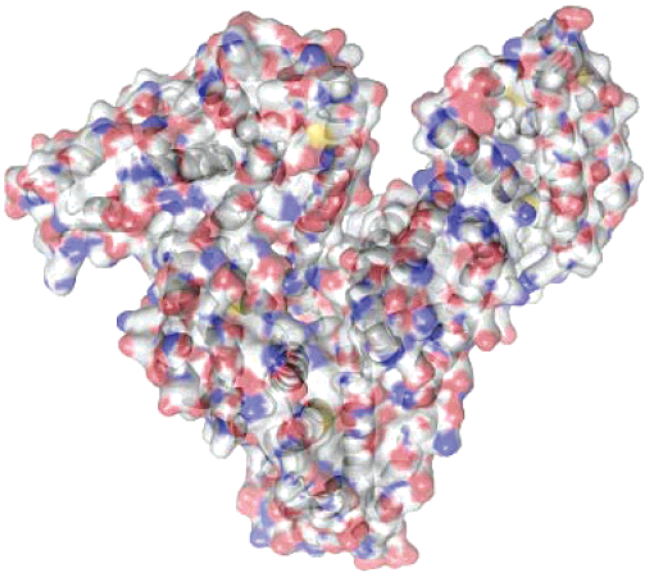

Figure 14. Human serum albumin complexed with myristate, phenylbutazone, and indomethacin deposited by Ghuman et al. (mmdbId: 35384; pdb: 2BXQ). Basic residues (arginine and lysine) are highlighted in blue. Acidic residues (aspartic acid and glutamic acid) are highlighted in red, and disulfide bridges are highlighted in yellow. Human serum albumin and bovine serum albumin (BSA) share a highly similar structure.

59 lysine groups present in the albumin molecule, 51 of them (∼86%) are found in the surface. Hence, most of the lysine groups in the albumin molecule are in its outer surface. Therefore, most of the tagged FITC molecule should be on the outer surface of the albumin molecule. Since a high concentration of FITC (10 mol of FITC per mol of albumin) is tagged to albumin through the positively charged lysine group, the surface of the albumin molecule might have become strongly electronegative due to suppression of the positive charge on the surface of albumin by FITC. On the other hand, TRITC could not have modified the surface charge of albumin as FITC did since only 1 mol of TRITC is tagged to 1 mol of albumin.

Conclusion

Tagging proteins with FITC might change their surface charge and thereby influence their interactions with other molecules including other proteins. Oppositely charged FITC tagged proteins form very sharp immobile interfaces in the gels. The interface is composed of positively charged aggregates that are trapped in the gel due to confinement imposed by the pore size of the gel. The relative positions of the interfaces are found to be independent of the pH of the buffer, and the formation of the interface is dependent upon a threshold stoichiometry of the interacting proteins. The formation of the aggregates is a result of modification of the surface of negatively charged proteins by FITC molecules. When a low concentration of poly-L-lysine was added to FITC albumin, a sharp decrease in the zeta potential value was observed, suggesting that poly-L-lysine favors the formation of macromolecular complexes having negatively charged FITC albumin on its surface. Higher concentrations of poly-L-lysine favor the formations of positively charged aggregates due to the presence of poly-L-lysine on its outer surface. The interface was formed by aggregates that were tightly bound and cannot be easily dissociated by the addition of NaCl. Although oppositely charged FITC tagged poly-L-lysine and FITC tagged albumin influence each others' migration velocities by forming aggregates, there was no observable secondary structural modifications in the amide I band regions when the proteins were mixed in solution.

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