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Fluorescence Labelling of Proteomic Samples

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Abstract— *Over the decades, covalent derivatization of proteins with fluorescent dyes prior to separation is widely used in the field of proteomic research. Our work examines the reactivity of a novel fluorescent dye with different proteins having different properties and check out for the reaction time of this dye with the proteins. Several experiments suggested that the reaction is increasing with time. Phosphate Buffer turned out to be a better option compared to Tris Buffer in terms of protein solubilization. Denaturing and non-denaturing gel electrophoresis suggested that the fluorescent dye reacts with Lysozyme, Bovine Serum Albumin and mammalian Red Fluorescent Protein but does not bind with Ubiquitin. Matrix assisted laser desorption/ionization-mass spectrometry also demonstrated in support of the gel electrophoresis results. The internal fluorescence property of the red fluorescent protein remained intact in the presence of this dye. This fluorescent dye can most likely be considered as a cysteine-specific dye. In many ways, fluorescence-labeling of cysteine residues presents a more attractive alternative than labeling any other residues. Serial dilution studies suggested that the sensitivity of this dye is high and can detect as low as 20 ng of BSA. Thereby, this novel fluorescent dye may serve as an alternative for labeling proteins where atleast one cysteine residue is present.*

Keywords— ***Fluorescence, reactivity, cysteine specific, sensitivity, proteomics.***

I. INTRODUCTION

Fluorescent detection of proteins has gained a lot of popularity in the fields of electrophoresis and proteomics (Rabilloud, 1990). The common aim of proteomics is to quantitatively define biological processes at the protein level, which typically involves the separation, display, and comparison of complex mixtures of proteins from reference and target cells, including protein concentrations spanning a broad range (Berggren et al., 2000; Urwin & Jackson, 1993). The common adsorptive staining methods used by most of us includes Coomassie blue, colloidal gold, and silver stain. However, these staining methods do not provide the requisite dynamic range, being linear over only a 10 to 40-fold difference in protein concentration, and suffers from being tedious and requiring harsh chemicals (Gee et al., 1996; Okeefe, 1994). On the other hand, fluorescence detection techniques, provide linear signal response over a much wider signal range of about three orders of magnitude than is found for the adsorptive dye alternatives mentioned above.

Fluorescent modification of proteins can be divided into two categories: covalent and noncovalent. In covalent methods, the proteins or proteomic samples are derivatized with a dye prior to electrophoresis or other separation methods. The main advantages of covalent derivatization methods for protein detection are the elimination of staining and distaining of gels, and the ability to image and capture the protein separation pattern immediately after electrophoresis (Briggs et al., 2000; Kapanidis & Weiss, 2002). In noncovalent methods, the proteins are first separated by SDS-PAGE followed by staining with dyes that bind to SDS-protein complexes (Rabilloud et al., 2001; Schuler, 2005).

Our main objective is to perform several experiments with a novel Fluorescent Dye and check whether this dye reacts/ binds with the different proteins. Also, if the reaction seems to occur between the dye and protein, we are keen to have a look at the reaction time. With the various advantageous applications which fluorescent staining is providing us, we want to check the sensitivity of this dye and show that this method of protein staining has greater advantages compared to any adsorptive method of protein staining.

II. MATERIALS AND METHODS

2.1 Preparation of Protein Samples

A certain concentration of pure proteins, namely, Ubiquitin (262 mM), Lysozyme (244 mM), Bovine Serum Albumin (326 mM), and mammalian Red Fluorescent Protein (65 mM) were purified. The proteins were buffer exchanged, if necessary, with either Tris (pH=8) or Phosphate Buffer (pH=8). For preparation of the protein samples, in order to set up the reaction mixture, each of the proteins were diluted with either Tris (pH=8) or Phosphate Buffer (pH=8) to 50 mM and stored at 4°C. ***Table 1*** represents the properties of the above-mentioned proteins used for reaction with the fluorescent dye.

Table 1: Basic Properties of Proteins used for reaction with the Fluorescent Dye

Proteins	*Molecular Weight (in kDa)*	*pI*	*No. of Cysteine residues*
Ubiquitin (His_6-tagged)	10.6	7.1	0
Lysozyme	14	11	10
BSA	68.7	4.5	35
m-RFP (His_6-tagged)	28.2	5.9	3

2.2 Preparation of the Fluorescent Dye

A yellow-colored powdered sample (~10 mg) was taken in an Eppendorf and dissolved in 500 mL of DMSO (Dimethyl sulfoxide). The dye was stored in 4°C and thawed every time before use. The structure of the dye molecule ***(Figure 1)*** predicts its molecular weight to be approximately 444 Da. Therefore, the stored concentration of the dye is 45 mM.

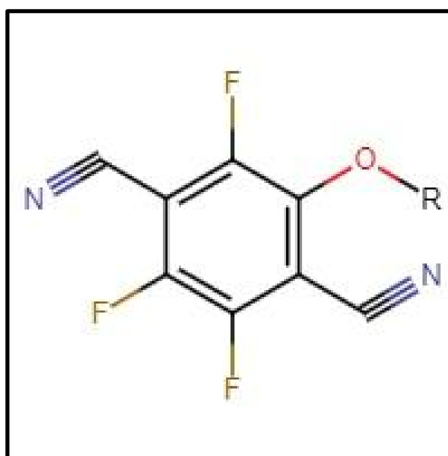


Fig.1: Structure of the Fluorescent Dye

2.3 Setting up of the Reaction Mixture

The reaction mixture was set up in 2 stages. Each of the protein-dye reaction was carried out in brown colored eppendorfs at room temperature.

i. 50 mL of 50 mM protein samples were collected in a brown eppendorf and to it was added 2 mL of 45 mM fluorescent dye, thereby, keeping the primary moles ratio of Protein:Dye = 1:36
ii. 20 mL of 50 mM protein samples were collected in a brown eppendorf and to it was added 2 mL of 4.5 mM fluorescent dye, thereby, keeping the primary moles ratio of Protein:Dye = 1:9

The reaction mixture was mixed well and kept aside. 4 mL of time samples were collected at minutes of 15, 30, 60, 120, 240 and 360. The reaction mixture was kept overnight at room temperature.

2.4 Dilution of Overnight Reaction Mixture

9 eppendorf tubes containing 30 mL of buffer (pH=8) was kept ready. To the first eppendorf (say eppendorf-1), 30 mL of the overnight reaction mixture was pipetted out and mixed in eppendorf-1. The reaction mixture can be said to be half diluted. In the similar fashion, 30 mL of the reaction mixture from eppendorf-1 was pipetted out and mixed in eppendorf-2. The process was continued till eppendorf-9 and 30 mL was discarded to maintain the volume. Therefore, samples were prepared in dilutions of 2^{-1} to 2^{-9} (***Figure 2***).

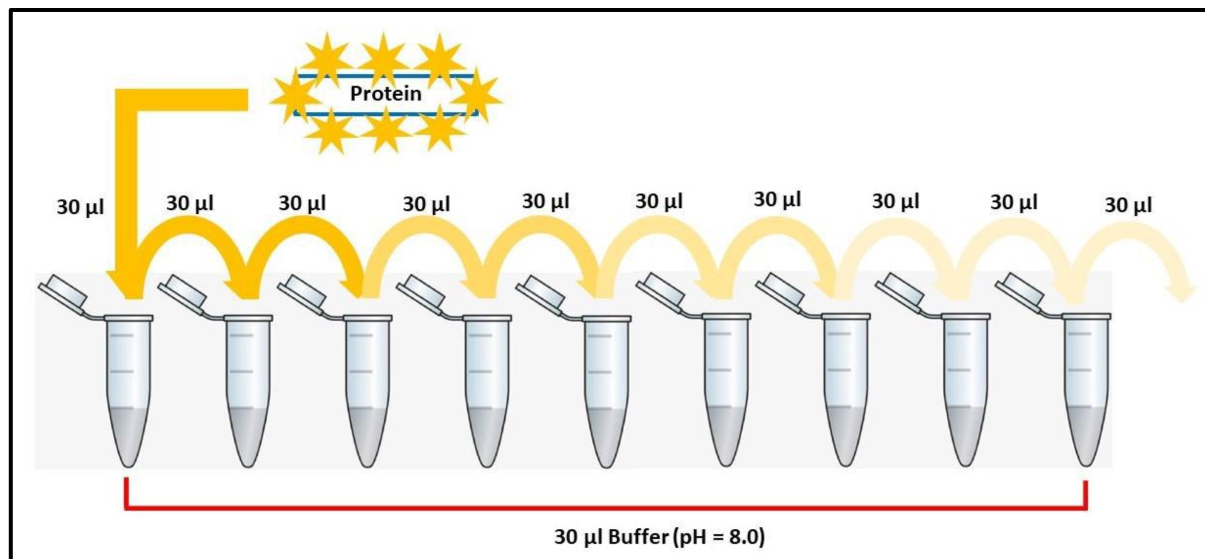


Fig.2: Process of serially diluting of overnight incubated Protein-Dye Reaction Mixture

2.5 Sample Preparation for SDS- Polyacrylamide Gel Electrophoresis and NATIVE- Polyacrylamide Gel Electrophoresis

For running an SDS-Page, reaction mixture was pipetted out and added to 20 mL of Tris Buffer (pH=8). To these was added 4X Bromophenol Blue Dye. The mixture was heated in a dry bath at 100°C for 5-10 minutes and spinned down using a table-top centrifuge. The samples were cooled for some time at 4°C and then loaded in the gel. The gel was run using Tris-Glycine Running Buffer at 100 V throughout.

For running a NATIVE-Page, reaction mixture was pipetted out and added to 20 mL of Tris Buffer (pH=8). To these was added 10X Tris Dye. The samples were incubated for about 30 minutes and then loaded in the gel. The gel was run using 0.5X TBE Buffer at 100 V throughout.

2.6 Observation of the Gel

After the gel was run at 100 V till the end, the plates were removed very carefully and the gel was placed on the Chemidoc plate for visualizing it under Fluorescein Blot Transilluminator plates. After which the gel was stained and destained in Coomassie Blue staining and destaining solutions respectively, followed by its observation on white tray under Protein gel Transilluminator.

2.7 Performing MALDI-Mass spectrometry

The MS samples were prepared by diluting the overnight reaction mixture with autoclaved water such that the sample does not contain anything more than 50 mM salt content. The protein concentration was maintained to about 2 mg/ml. The samples were then mixed with sinapic acid, the matrix used for MS of proteins, and MALDI-Mass Spectrometry was performed.

III. RESULTS AND DISCUSSION

3.1 Experiments with Ubiquitin

100 mL of 50 mM pure Ubiquitin was taken in a brown eppendorf to provide a dark environment to the Ubiquitin-Dye reaction. Keeping the primary moles ratio of Ubiquitin:Dye = 1:36, 4 mL of 45 mM Fluorescent Dye was mixed to 100 mL of Ubiquitin. 1 mL of reaction mixture was collected at time intervals of 15, 30, 60, 120, 240 and 360 minutes, and added to 20 mL of Tris Buffer (pH=8) and 4X Brilliant Blue Dye. The samples were then run on 12% SDS-Page. On observing the gel image under UV Transilluminator for Fluorescein blots, we cannot comment appropriately on the reaction time or whether the dye reacts

with Ubiquitin or not. Therefore, the brighter bands which we are observing may either be of the Ubiquitin-Dye complex or the free Dye ***(Figure 3a).***

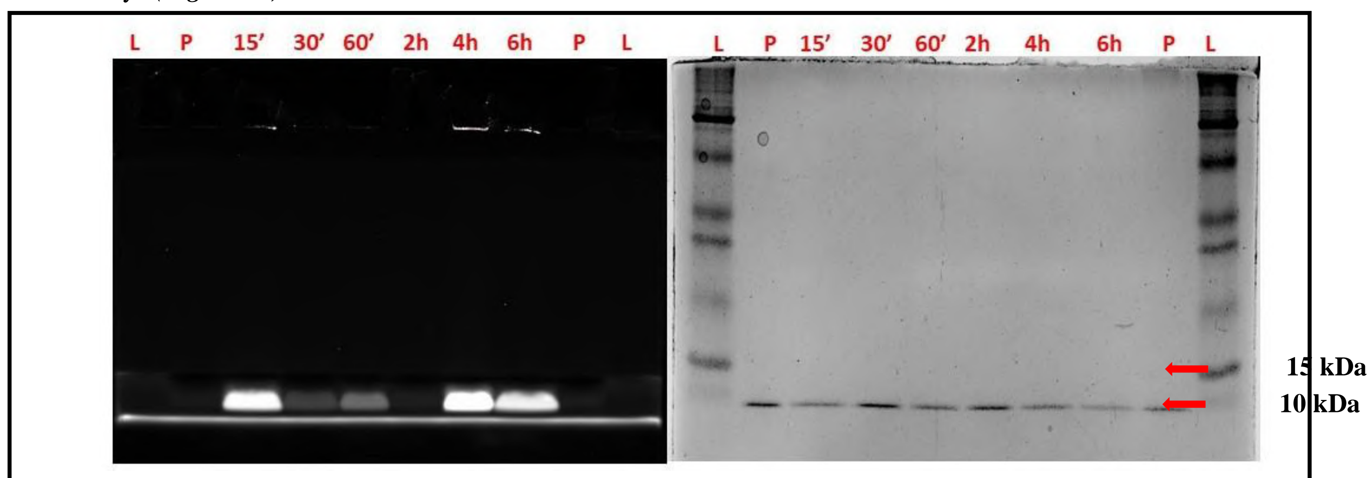


Fig.3a: SDS-Page Gel Images of the time samples observed under Fluorescein Blots and Protein Gel Transilluminator. L = Ladder; P = 50 mM Ubiquitin; 15', 30', 60' = sample collected in minutes; 2h, 4h, 6h = sample collected in hours

To verify the doubt about the bright bands which we are observing just above the dye front, two experiments were performed after keeping the reaction mixture for overnight incubation at room temperature: (i) running of 8% NATIVE-Page ***(Figure 3b)***, and (ii) performing MALDI- Mass spectrometry ***(Figure 3c).*** From both the above-mentioned experiments, which we performed, we can tell that Ubiquitin is not reacting with the dye.

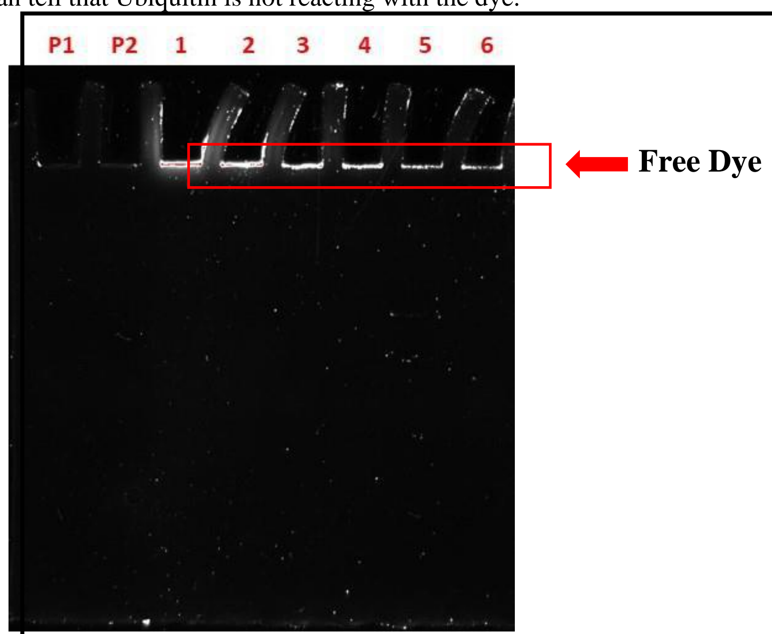
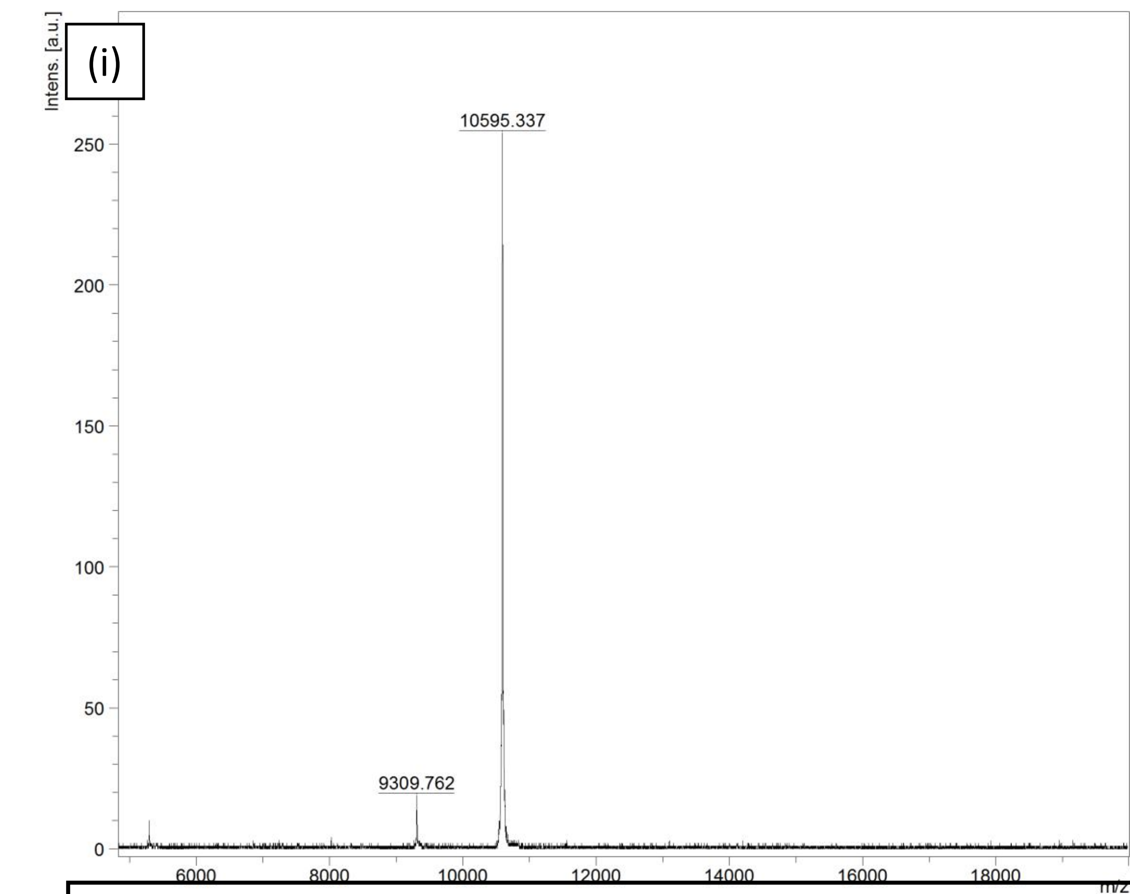


Fig.3b: NATIVE-Page Gel Image of the diluted overnight samples observed under Fluorescein Blots. P1 = 50 mM Ubiquitin; P2 = 25 mM Ubiquitin; 1, 2, 3, 4, 5, 6 = diluted samples of overnight reaction mixture (less diluted to high diluted). The bright bands on the gel are of the unreacted (free) dye. No bands of Ubiquitin-Dye observed.


(i)
Intens. [a.u.]
10595.337
9309.762
250
200
150
100
50
0
6000
8000
10000
12000
14000
16000
18000
m/z

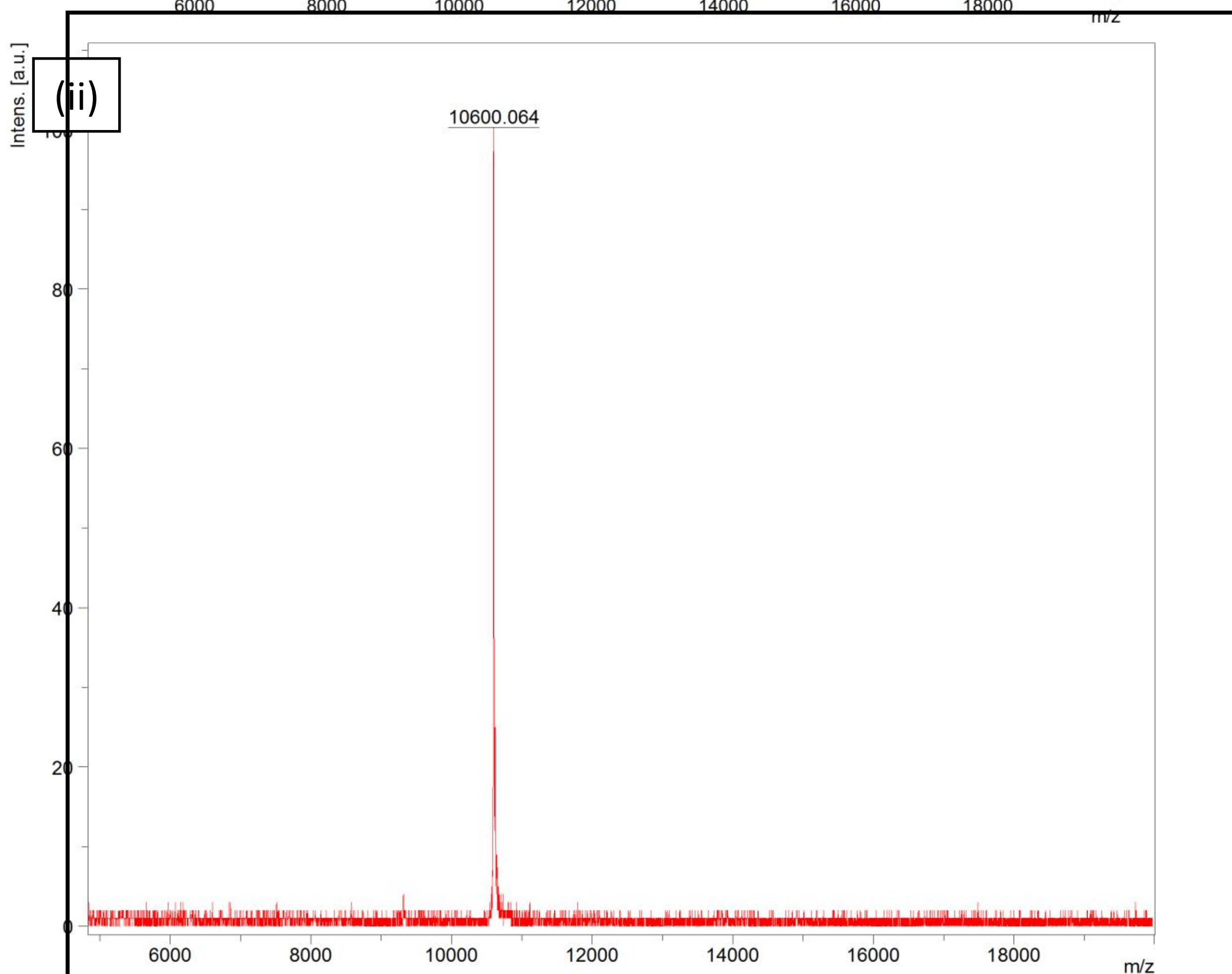

(ii)
Intens. [a.u.]
10600.064
80
60
40
20
0
6000
8000
10000
12000
14000
16000
18000
m/z

Fig.3c: MALDI-MS of: (i) pure His-tagged Ubiquitin and (ii) Ubiquitin-Dye complex. Mass spectrometry suggested that Ubiquitin (M.W. = 10595.337 Da) and Ubiquitin-Dye complex (M.W. = 10600.064 Da) have no significant change in mass/charge value, hence there is no possibility of the Dye (M.W. = 444 Da) to have reacted with Ubiquitin.

3.2 Experiments with Lysozyme

50 mL of 50 mM pure Lysozyme was taken in a brown eppendorf to provide a dark environment to the Lysozyme-Dye reaction. Keeping the primary moles ratio of Lysozyme:Dye = 1:36, 2 mL of 45 mM Fluorescent Dye was mixed to 50 mL of Lysozyme. 4 mL of reaction mixture was collected at time intervals of 15, 30, 60 and 120 minutes, and added to 20 mL of Tris Buffer (pH=8) and 4X Brilliant Blue Dye. The samples were then run on 12% SDS-Page. On observing the gel image under UV Transilluminator, we failed to comment appropriately on the reaction time and also the feasibility of the reaction. The brighter bands which we are observing may either be of the Lysozyme-Dye complex or the free Dye ***(Figure 4a).***

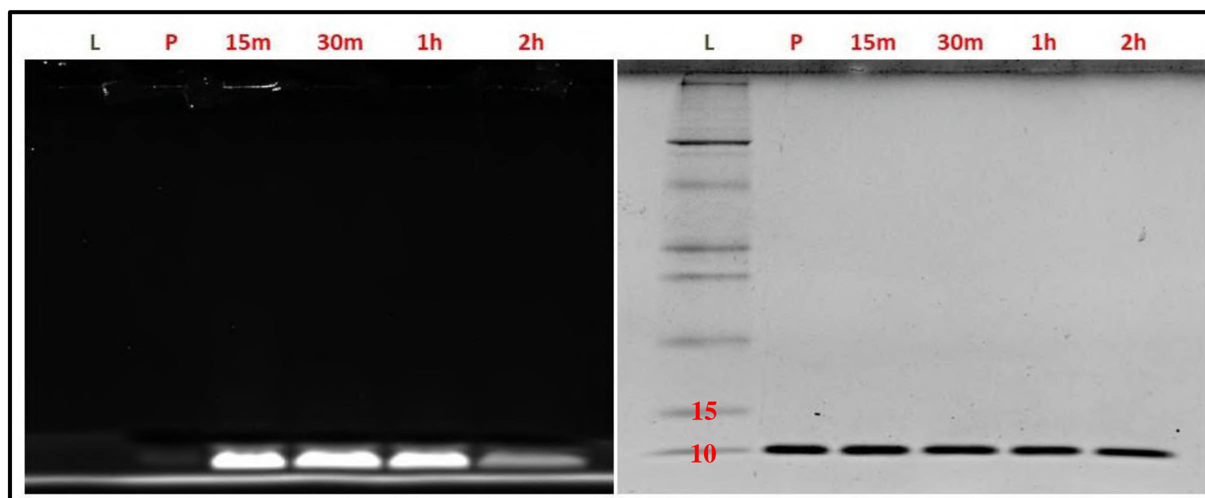


Fig.4a: SDS-Page Gel Images of the time samples observed under Fluorescein Blots and Protein Gel Transilluminator. L = Ladder; P = 50 mM Lysozyme; 15', 30' = sample collected in minutes; 1h, 2h = sample collected in hours

To verify the doubt about the bright bands which we are observing just above the dye front, we ran 8% NATIVE-Page ***(Figure 4b).*** On observing the gel under UV Transilluminator, we can tell that Lysozyme does react with the dye.

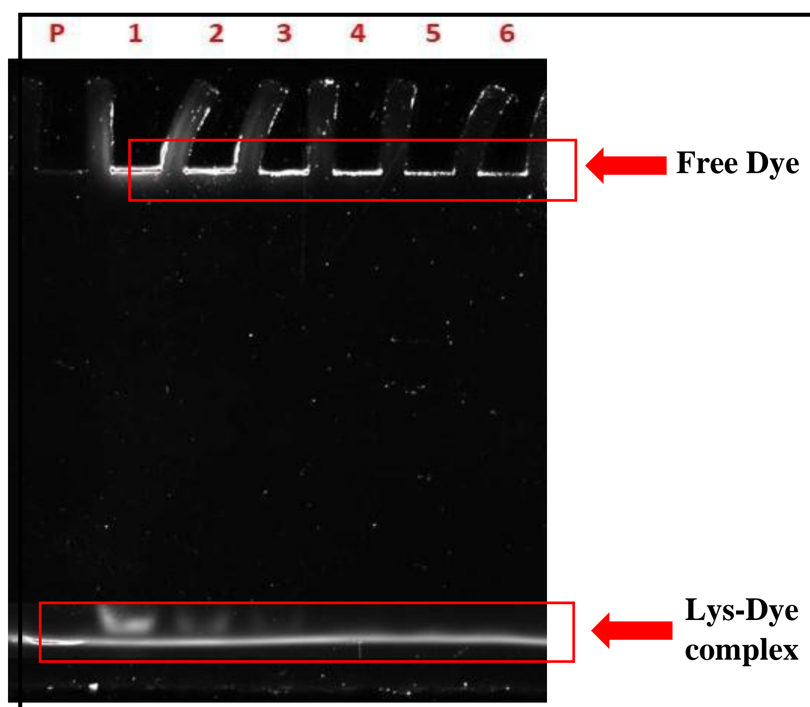


Fig.4b: NATIVE-Page Gel Image of the diluted overnight samples observed under Fluorescein Blots. P = 50 mM Lysozyme; 1, 2, 3, 4, 5, 6 = diluted samples of overnight reaction mixture (less diluted to high diluted). The bright bands on the gel above the wells are of the free dye whereas the bright bands just above the dye front are of the Lysozyme-Dye complex.

3.3 Experiments with Bovine Serum Albumin

50 mL of 50 mM BSA was taken in a brown eppendorf to provide a dark environment to the BSA-Dye reaction. Keeping the primary moles ratio of BSA:Dye = 1:36, 2 mL of 45 mM Fluorescent Dye was mixed to 50 mL of BSA. 4 mL of reaction mixture was collected at time intervals of 15, 30, 60 and 120 minutes, and added to 20 mL of Tris Buffer (pH=8) and 4X Brilliant Blue Dye. The samples were then run on 12% SDS-Page. On observing the gel image under UV Transilluminator, we could see very faint bands ***(Figure 5a).*** When the buffer was changed from Tris to Phosphate (pH=8), we could see better intensified bands near the 69 kDa Molecular weight marker ***(Figure 5b).*** The brighter bands which we are observing just above the dye front is of the free Dye.

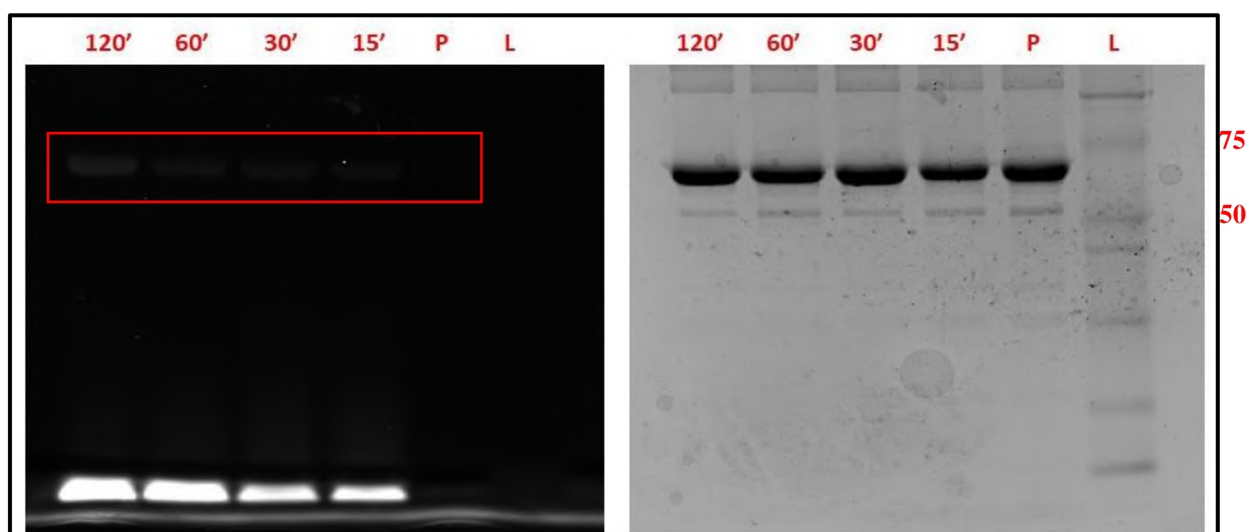


Fig.5a: Bovine Serum Albumin in Tris Buffer (pH=8) and mole ratio of BSA:Dye = 1:36. SDS-Page Gel Images of the time samples observed under Fluorescein Blots and Protein Gel Transilluminator. L = Ladder; P = 50 mM BSA; 15', 30', 60',

120' = sample collected in minutes. The bright bands on the gel above the dye front are of the free dye whereas the bright bands at 69 kDa are of the BSA-Dye complex.

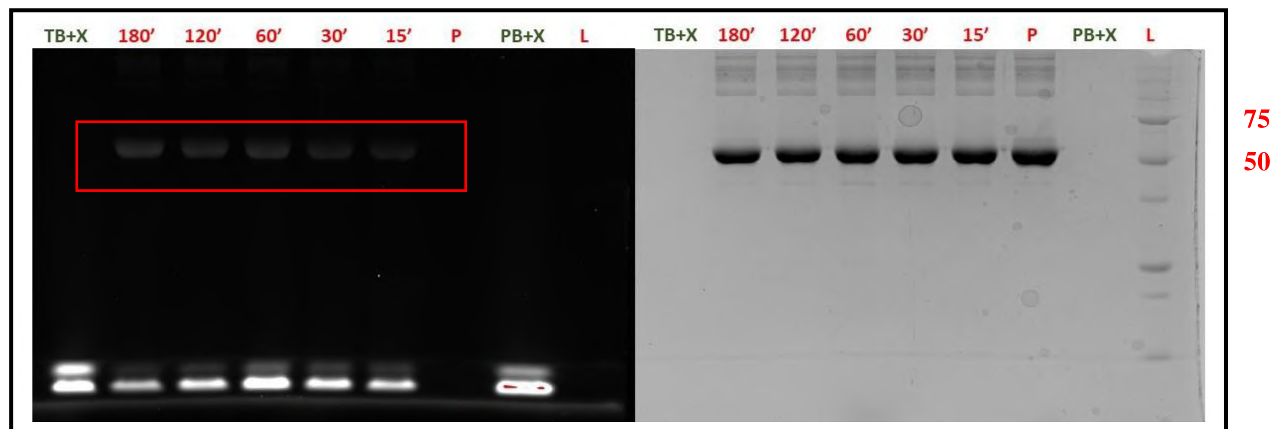


Fig.5b: Bovine Serum Albumin in Phosphate Buffer (pH=8) and mole ratio of BSA:Dye = 1:36. SDS-Page Gel Images of the time samples observed under Fluorescein Blots and Protein Gels. Two control experiments were also set i.e., Tris Buffer and Phosphate Buffer mixed with Fluorescent Dye. L = Ladder; P = 50 mM BSA; 15', 30', 60', 120', 180' = sample collected in minutes; TB+X = Tris Buffer + Dye; PB+X = Phosphate Buffer + Dye. The bright bands on the gel above the dye front are of the free dye whereas the bright bands in the middle are of the BSA-Dye complex.

9 Eppendorf tubes containing 30 mL of Phosphate buffer (pH=8) was kept ready. To eppendorf-1, 30 mL of the overnight reaction mixture was pipetted out and mixed well. The BSA-Dye mixture is now half diluted. Following the same process, 30 mL of the reaction mixture from eppendorf-1 was pipetted out and mixed in eppendorf-2 to dilute it further. The process was continued till eppendorf-9 and the remaining 30 mL was discarded to maintain the volume. Therefore, samples were prepared in dilutions of 2^{-1} to 2^{-9}. The samples were incubated in dry bath for 10 minutes and SDS-Page was run followed by its observation under UV Transilluminator ***(Figure 5c)***. Bright bands at around 66 kDa assures the fluorescent dye to be reacting with Bovine Serum Albumin. Running of the diluted BSA-Dye mixture on 8% NATIVE-Page ***(Figure 5d)*** and performing MALDI-MS ***(Figure 5e)*** also speaks in its support.

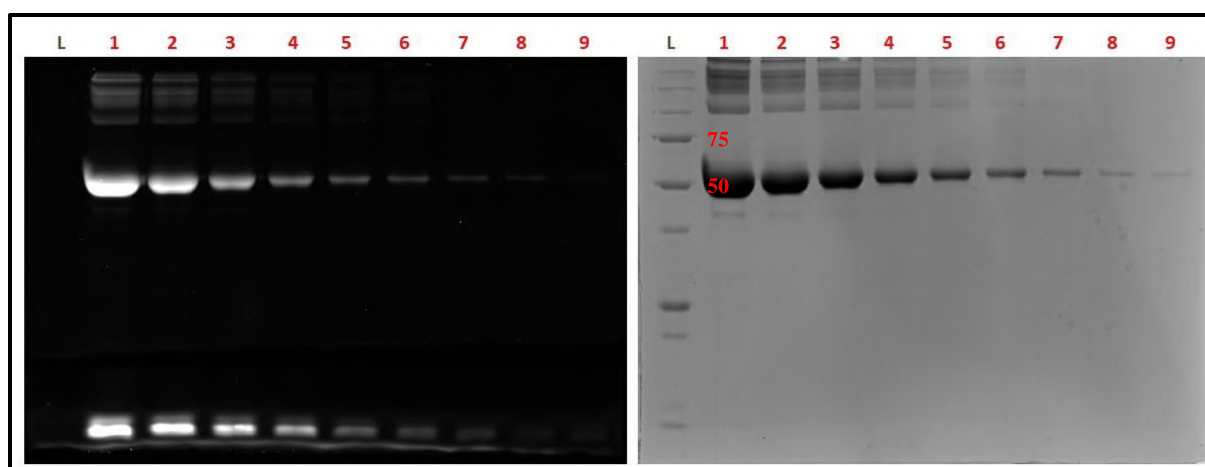


Fig.5c: Serial dilutions of Bovine Serum Albumin-Fluorescent Dye in Phosphate Buffer (pH=8). SDS-Page Gel Images of the dilution samples of BSA-Dye complex observed under Fluorescein Blots and Protein Gel Transilluminator. L = Ladder; 1-9 = amount of BSA present in the eppendorf tubes (1: 12.3 µg; 2: 6.15 µg; 3: 3.08 µg; 4: 1.54 µg; 5: 769 ng; 6: 384 ng; 7: 192 ng; 8: 96 ng; 9: 48 ng)

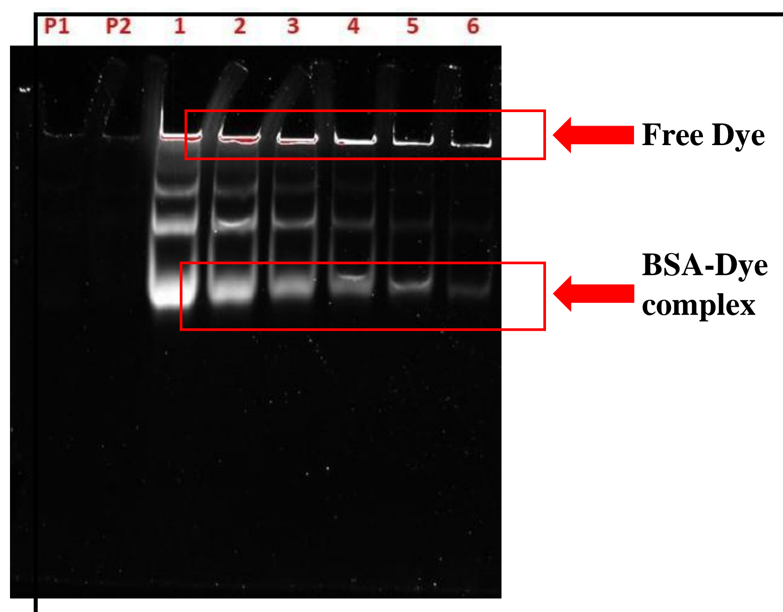


Fig.5d: NATIVE-Page Gel Image of the diluted overnight samples observed under Fluorescein Blots. P1 = 50 mM BSA; P2 = 25 mM BSA; 1, 2, 3, 4, 5, 6 = diluted samples of overnight reaction mixture (less diluted to high diluted). The bright bands on the gel are of the BSA-Dye complex. The bright bands on the gel above the wells are of the free dye whereas the bright bands in the middle are of the BSA-Dye complex.

(i)
Intens. [a.u.]
68686.114
61815.668
5
4
3
2
1
0
25000 30000 35000 40000 45000 50000 55000 60000 65000 70000
m/z

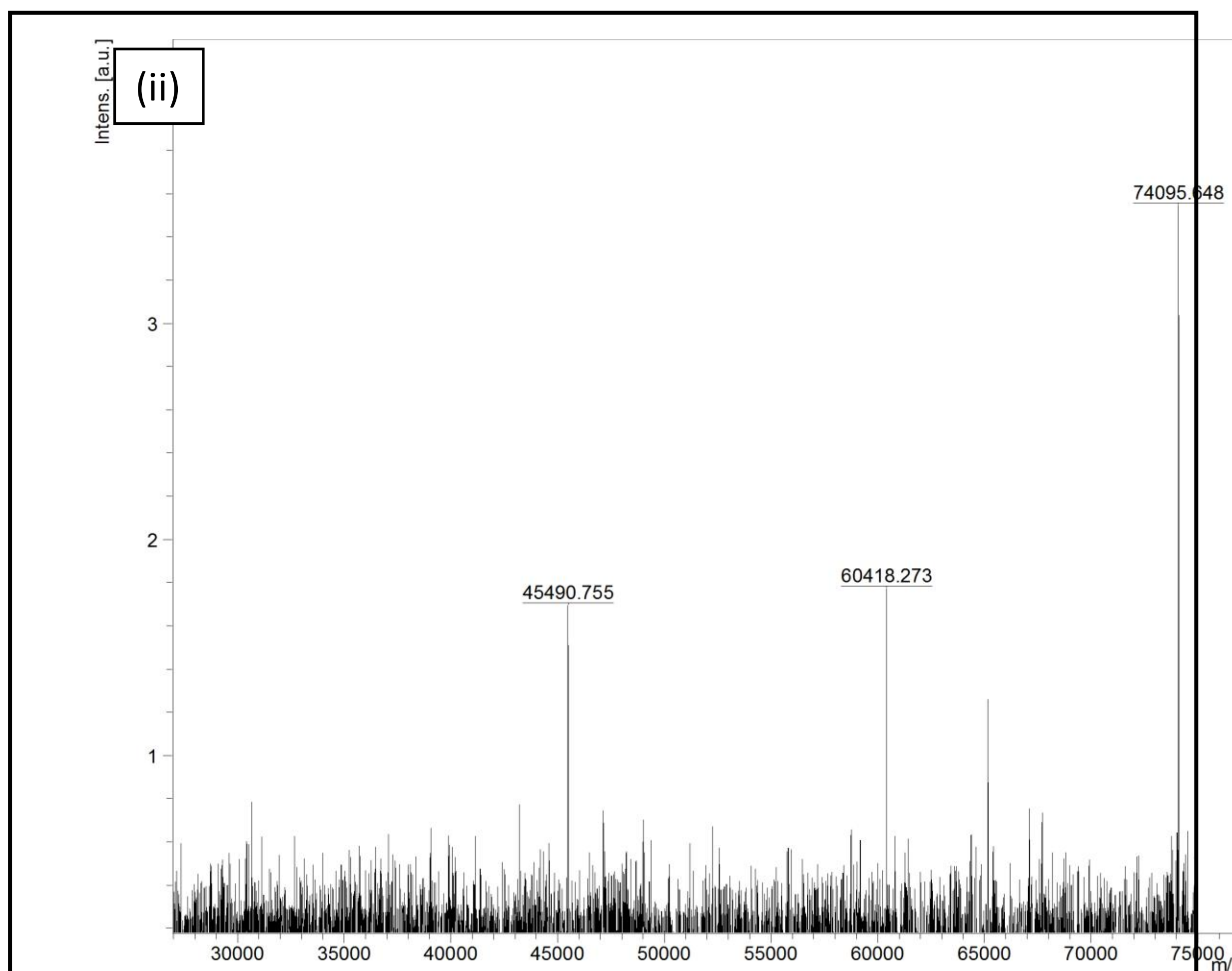


Fig.5e: MALDI-MS of: (i) pure BSA and (ii) BSA-Dye complex. Mass spectrometry suggested that BSA (M.W. = 68686.114 Da) and Ubiquitin-Dye complex (M.W. = 74095.648 Da) have a difference (of 5409.534 Da) in the mass/charge value and therefore we can conclude that the Dye (M.W. = 444 Da) is reacting with BSA.

Keeping an eye on the sensitivity of the Fluorescent Dye, we wanted to check how much diluted concentration of proteins and dye would be sufficient for visualization. In order to check this, the mole ratio of BSA:Dye was lowered from 1:36 to 1:9. 20 mL of 50 mM BSA was taken in a brown eppendorf to provide a dark environment to the reaction. Keeping the primary moles ratio of BSA:Dye = 1:9, 2 mL of 4.5 mM Fluorescent Dye was mixed to 50 mL of BSA. 4 mL of reaction mixture was collected at time intervals of 15, 30, 60, 120, 180, 240, 300 and 360 minutes, and added to 20 mL of Tris Buffer (pH=8) and 4X Brilliant Blue Dye. The samples were then run on 12% SDS-Page. On observing the gel image under UV Transilluminator, we could see very faint bands on the line of our protein ***(Figure 5e)*** **.** On changing the buffer from Tris to Phosphate (pH=8), the bands intensified at 69 kDa ***(Figure 5f).*** So, Phosphate Buffer (pH=8) could be a better option for performing reactions with this Fluorescent Dye. We can comment that with increasing time, the protein-dye complex bands seem to intensify, thereby, indicating the increasing binding of the dye to the proteins.

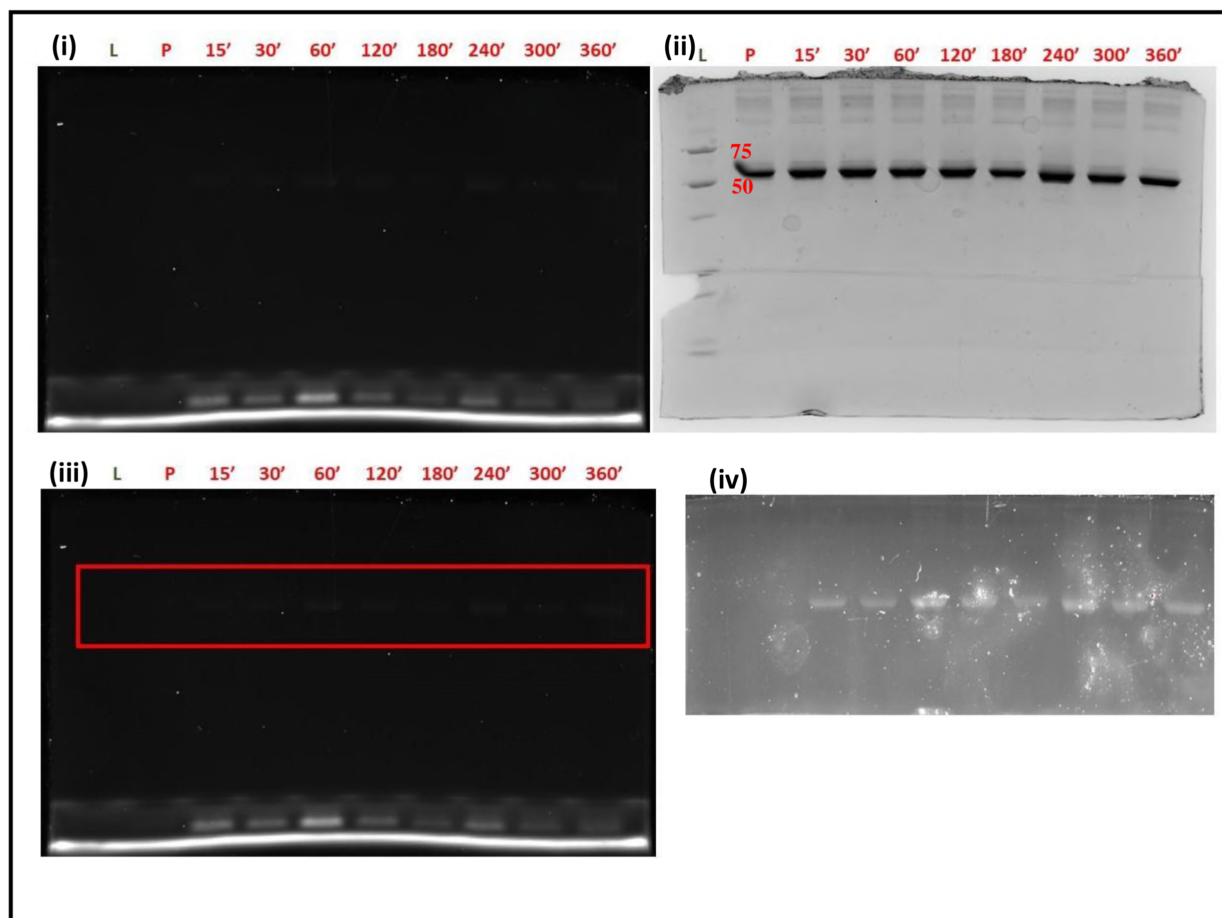


Fig.5e: Bovine Serum Albumin in Tris Buffer (pH=8) and mole ratio of BSA:Dye = 1:9. SDS-Page Gel Images of the time samples observed under (i), (iii), (iv) Fluorescein Blots and (ii) Protein Gel Transilluminator. Two control experiments were also set i.e., Tris Buffer mixed with Fluorescent Dye and Phosphate Buffer mixed with Fluorescent Dye. L = Ladder; P = protein; 15', 30', 60', 120', 180', 240', 300', 360' = sample collected in minutes. On intensifying the selected region of (iii), we can tell from (iv) that with increasing time, the binding of dye to the protein increases.

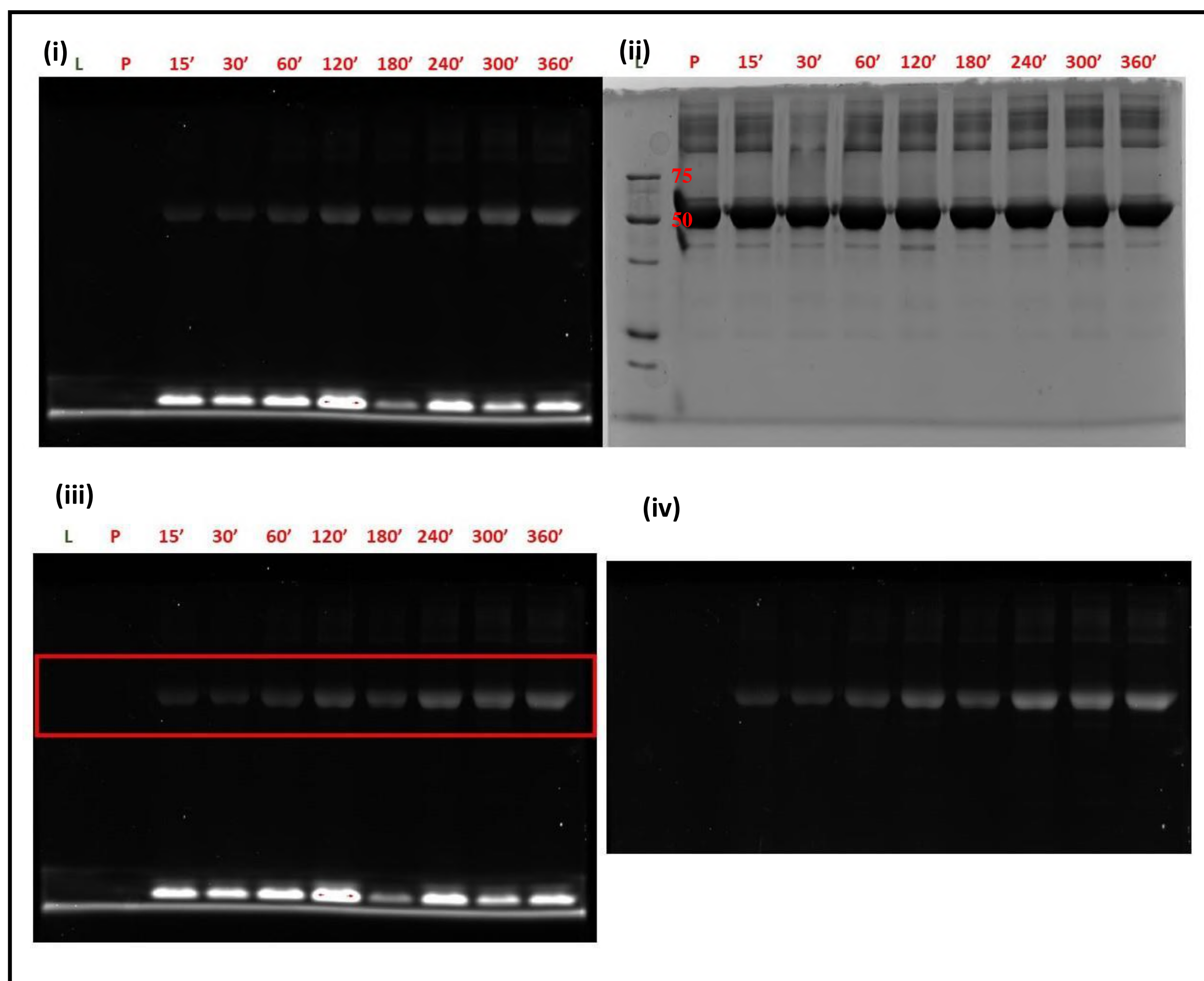


Fig.5f: Bovine Serum Albumin in Phosphate Buffer (pH=8) and mole ratio of BSA:Dye = 1:9. SDS-Page Gel Images of the time samples observed under (i), (iii), (iv) Fluorescein Blots and Protein Gel Transilluminator. Two control experiments were also set i.e., Tris Buffer mixed with Fluorescent Dye and Phosphate Buffer mixed with Fluorescent Dye. L = Ladder; P = protein; 15', 30', 60', 120', 180', 240', 300', 360' = sample collected in minutes. On intensifying the selected region of (iii), we can tell from (iv) that with increasing time, the binding of dye to the protein increases.

9 eppendorf tubes containing 30 mL of Tris buffer (pH=8) was kept ready. To eppendorf-1, 30 mL of the overnight reaction mixture was pipetted out and mixed well. The BSA-Dye mixture is now half diluted. Following the same process, 30 mL of the reaction mixture from eppendorf-1 was pipetted out and mixed in eppendorf-2 to dilute it further. The process was continued till eppendorf-9 and the remaining 30 mL was discarded to maintain the volume. Therefore, samples were prepared in dilutions of 2^{-1} to 2^{-9}. The samples were incubated in dry bath for 10 minutes and SDS- Page was run followed by its observation under UV Transilluminator ***(Figure 5g)***. The entire experiment of serial dilutions were performed in Phosphate Buffer (pH=8) as well ***(Figure 5h)*** followed by visualization. We can also tell from the gel images that the sensitivity of this dye lies somewhat in between 38.5 ng to 19.5 ng of proteins with is much more sensitive compared to any other adsorptive protein staining methods.

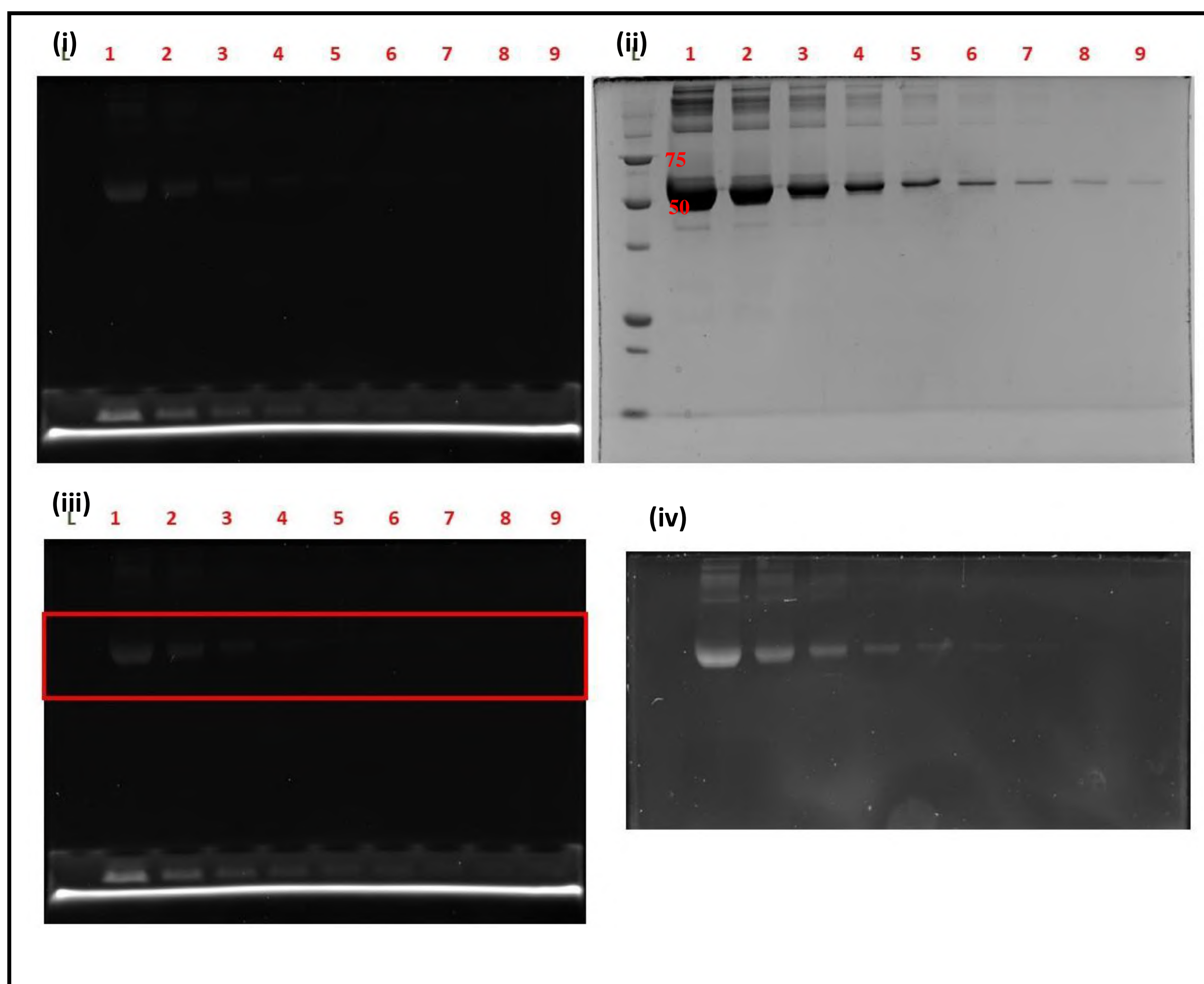


Fig.5g: Serial dilutions of Bovine Serum Albumin-Fluorescent Dye in Tris Buffer (pH=8). SDS-Page Gel Images of the dilution samples of BSA-Dye complex observed under (i), (iii), (iv) Fluorescein Blots and (ii) Protein Gel Transilluminator. L = Ladder; 1-9 = amount of BSA present in the eppendorf tubes (1: 4.92 µg; 2: 2.46 µg; 3: 1.23 µg; 4: 615 ng; 5: 308 ng; 6: 154 ng; 7: 77 ng; 8: 38.5 ng; 9: 19.25 ng). On deep analysis from the selected region of (iii), we can tell from (iv) that the sensitivity of the dye is somewhat in between 19.5 ng - 38.5ng.

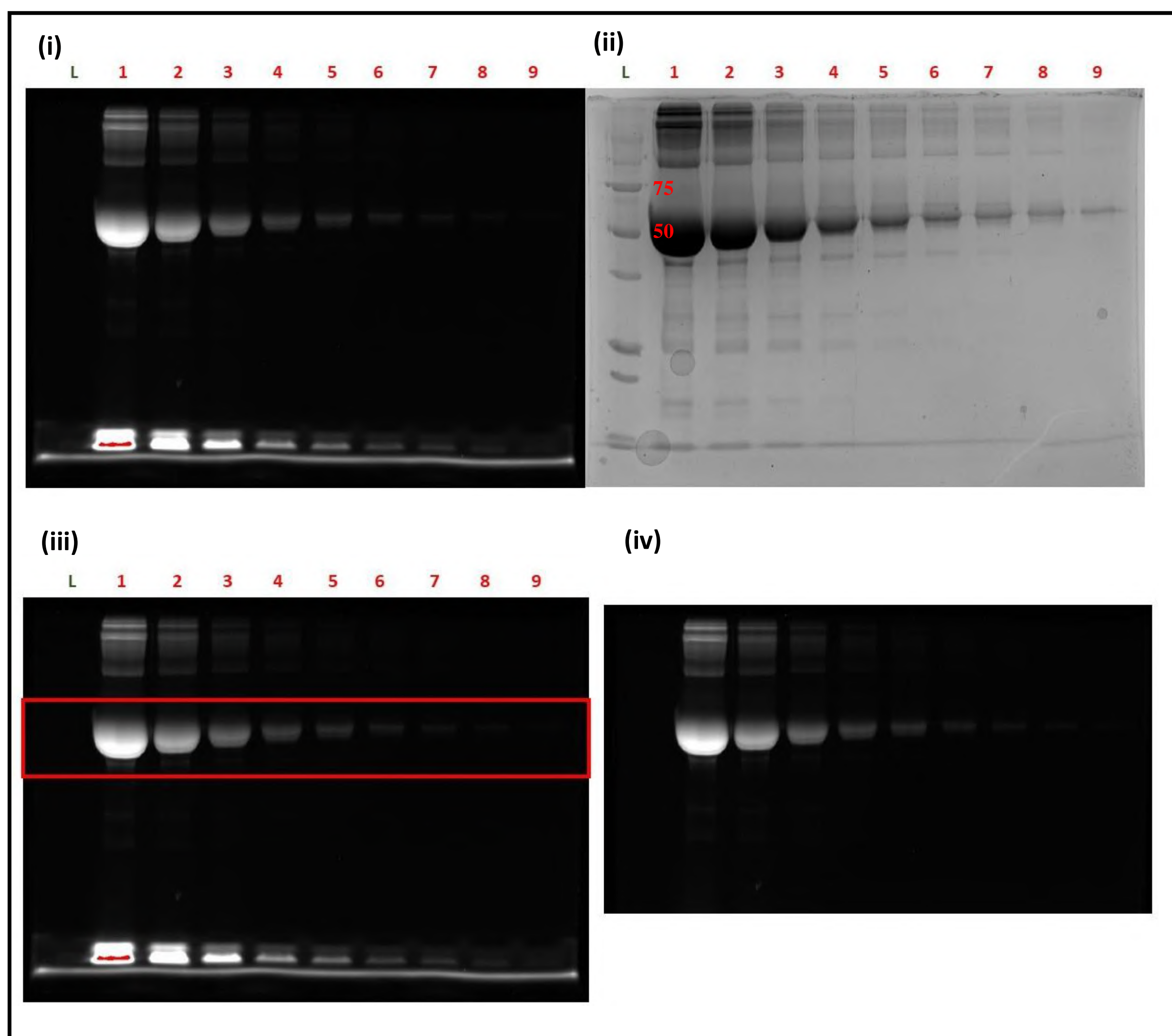


Fig.5h: Serial dilutions of Bovine Serum Albumin-Fluorescent Dye in Phosphate Buffer (pH=8). SDS-Page Gel Images of the dilution samples of BSA-Dye complex observed under (i), (iii), (iv) Fluorescein Blots and (ii) Protein Gel Transilluminator. L = Ladder; 1-9 = amount of BSA present in the eppendorf tubes (1: 4.92 µg; 2: 2.46 µg; 3: 1.23 µg; 4: 615 ng; 5: 308 ng; 6: 154 ng; 7: 77 ng; 8: 38.5 ng; 9: 19.25 ng). On deep analysis from the selected region of (iii), we can tell from (iv) that the sensitivity of the dye is somewhat in between 19.5 ng - 38.5ng.

3.4 Experiments with mammalian Red Fluorescent Protein

50 mL of 50 mM m-RFP was taken in a brown eppendorf to provide a dark environment to the BSA-Dye reaction. Keeping the primary moles ratio of mRFP:Dye = 1:9, 1 mL of 22.5 mM Fluorescent Dye was mixed to 50 mL of m-RFP. The reaction setup was kept overnight for about 12 hours. 9 eppendorf tubes containing 30 mL of Tris buffer (pH=8) was kept ready. To eppendorf-1, 30 mL of the overnight reaction mixture was pipetted out and mixed well. The mRFP-Dye mixture is now half diluted. Following the same process, 30 mL of the reaction mixture from eppendorf-1 was pipetted out and mixed in eppendorf-2 to dilute it further. The process was continued till eppendorf-9 and the remaining 30 mL was discarded to maintain the volume. Therefore, samples were prepared in dilutions of 2^{-1} to 2^{-7}. The samples were incubated in dry bath for 10 minutes and SDS-Page was run followed by its observation under UV Transilluminator ***(Figure 6a)***. Bright bands at around 28.2 kDa assures the fluorescent dye to be reacting with mammalian Red Fluorescent Protein. However, we are also observing a fluorescence of the m-RFP (without dye) under Fluorescein Blots as well as under the RFP channel, which we did not expect in the denaturing gel. Use of SDS should have unfolded the protein and the internal fluorescence property of m-RFP should have been lost. But we don't see this, probably, due to its robust property.

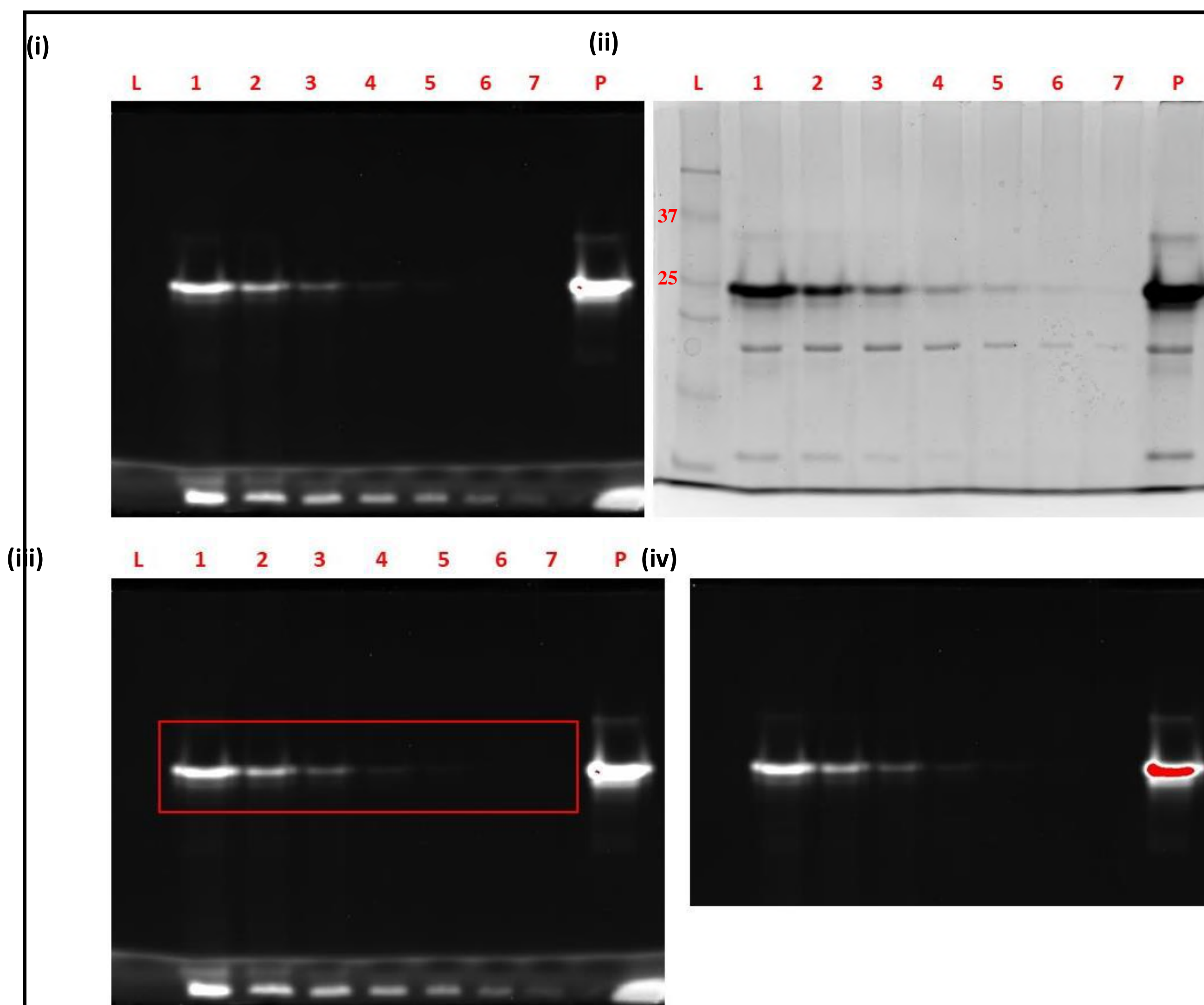


Fig.6a: Serial dilutions of Mammalian RFP-Fluorescent Dye in Tris Buffer (pH=8). SDS-Page Gel Images of the dilution samples of mRFP-Dye complex observed under Fluorescein Blots and Protein Gel Transilluminator. L = Ladder; P = protein; 1-7 = diluted samples of overnight reaction mixture (less diluted to high diluted). The bright bands on the gel are of the mRFP-Dye complex.

In order to verify the reactivity of the dye with m-RFP, running of the overnight mRFP-Dye mixture on 8% NATIVE-Page ***(Figure 6b)*** was required. 7 eppendorf tubes containing 30 mL of Tris buffer (pH=8) was kept ready. To eppendorf-1, 30 mL of the overnight reaction mixture was pipetted out and mixed well. Next, 30 mL of the reaction mixture from eppendorf-1 was pipetted out and mixed in eppendorf-2 to dilute it further. The process was continued till eppendorf-9 and the remaining 30 mL was discarded to maintain the volume. Eppendorf-0 only contained 30 mL of the overnight reaction mixture (without Buffer). Therefore, samples were prepared in dilutions of 2^0 to 2^{-7}. The samples were incubated in dry bath for 10 minutes and SDS-Page was run followed by its observation under RFP channel ***[Figure 6b(i)]*** and Fluorescein channel ***[Figure 6b(ii)]***. We can observe that the intensity of the gel image that was obtained under Fluorescein channel seems to be less intensified than the one obtained under RFP channel. This indicates that although, we are observing the protein band on the gel which is due to its internal property, we are also observing brighter bands for the diluted mixture under the RFP channel and henceforth if we subtract the band intensity of gel observed under Fluorescein channel from the RFP channel, we can get the actual intensity of the mRFP-Dye complex. However, coming to our main point of interest, we can tell that the mammalian Red Fluorescent

Protein is reacting with the fluorescent dye. And also, we got to know that even in the presence of the fluorescent dye, the internal fluorescence of the proteins are not disrupted.

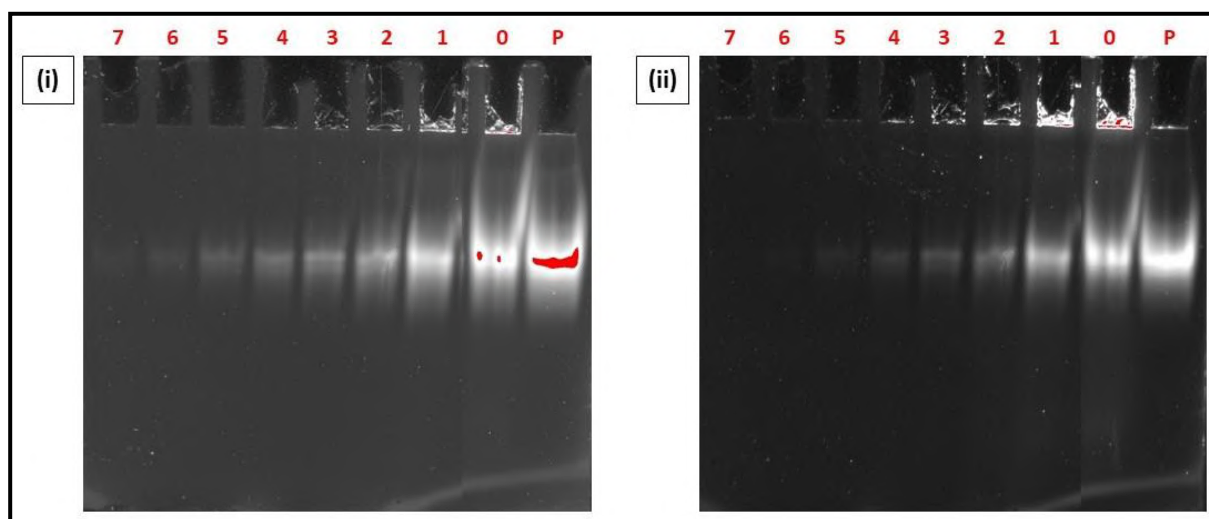


Fig.6b: NATIVE-Page Gel Images of mRFP-Fluorescent Dye in Tris Buffer (pH=8). (i) Gel Image of the overnight dilution samples of mRFP-Dye complex observed under RFP channel; (ii) Gel Image of the overnight dilution samples of mRFP-Dye complex observed under Fluorescein channel; P = protein; 0-7 = amount of mRFP present in the eppendorf tubes (0: 7.4 µg; 1: 3.7 µg; 2: 1.85 µg; 3: 925 µg; 4: ng; 5: 550 ng; 6: 275 ng; 7:138 ng)

IV. CONCLUSION

Over the decades, fluorescent method of protein detection has gained popularity and has greater advantages compared to any other protein staining method. In order to perform several experiments with the novel Fluorescent Dye, we selected different proteins namely, Ubiquitin, Lysozyme, Bovine Serum Albumin and mammalian Red Fluorescent Protein. After performing SDS and NATIVE Polyacrylamide Gel Electrophoresis followed by MALDI-MS, we could confirm from the results that the fluorescent dye reacts with Lysozyme (10 cysteines), Bovine Serum Albumin (35 cysteines) and mammalian Red Fluorescent Protein (3 cysteines) but does not show any binding with Ubiquitin (no cysteines). Hence, this fluorescent dye is most likely to be a cysteine-specific dye. We could also tell from the time sample experiments that Phosphate Buffer (pH=8) is a better option while reacting with the dye. Commenting on the reaction time, we received sufficient evidences of bands intensifying with increasing time. However, the time of completion of the reaction is not yet established but letting the protein to react with the dye for an hour or so would be sufficient for visualization. Studies have shown that at least 45-50 ng of protein is required for adsorptive staining dyes to sense proteins. Serial dilution studies predict the sensitivity of the fluorescent dye to be around <30 ng of BSA. Henceforth, we can consider this novel fluorescent dye to be a better alternative for cysteine-specific protein staining.

V. FUTURE PERSPECTIVES

1) To find out how many molecules of the Fluorescent Dye is actually binding to the Proteins. To carry out this objective, we can perform MALDI-MS and MS/MS followed by NMR Spectrometry
2) Although we are assuming Cysteines to be the most probable site for binding of the dye, yet we need to appropriately locate its binding site which might either be Lysine, Threonine, Serine or N-terminal end,
3) To perform the reaction of the Protein and Fluorescent Dye at different conditions such as:
    a) Using same concentration of Proteins with different concentrations of BME which would break the disulphide bonds.
    b) Carrying out the reaction over a wide range of pH which will bring about destabilization in the formation of ester, thio-ester, and amide bonds.

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