University of Toronto

Faculty of Applied Science and Engineering

BME440 Biomedical Engineering Technology and Investigation

Lab 2: Protein Binding & Native PAGE EMSA

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1.0 Introduction & scientific objective

Gel electrophoresis is a commonly used laboratory technique that aims to separate charged biomacromolecules based on their size, shape and charge, by applying an electric field to a gel in which the protein of interest is added. Native PAGE EMSA is a type of this technique and it is used to visually detect protein interactions with other biomolecules such as other proteins, DNA and RNA. [2] In EMSA, a porous gel, such as polyacrylamide or agarose, is used to observe the rate of movement of molecules of interest through the gel when an electric current is applied. By comparing the relative shift of the protein complex that is bound to another biomacromolecule versus the protein itself in the gel, we can obtain information about association and dissociation kinetics, binding stoichiometry and epitope identification of the proteins of interest. [1]

The scientific problem that will be addressed in this lab is how to qualitatively determine the binding properties of a protein with unknown affinity to another biomacromolecule. The objective of this lab is to observe the binding stoichiometry of DNA-based aptamer to the protein Thrombin by using Native PAGE EMSA. Through using this experiment, we expect to observe that the aptamer-thrombin complexes with higher protein:nucleic acid ratio creates darker, shifted bands in comparison to free aptamer, indicating the formation of more complexes.

2.0 Materials and Methods

In this lab, we incubated our 10 μM TBA Aptamer sample in a 95°C hot water bath for 5 minutes. We then prepared a serial dilution series of a 38.4 μM Thrombin solution and added 10 μL of the Aptamer Binding Reaction Buffer (100 mM KCl) into each tube. Once our TBA Aptamer reached room temperature, we added 2 μL of it to each tube, except the Thrombin Control sample. After incubating for 30 minutes, we prepared the PAGE Gel Electrophoresis Apparatus and added 4 μL of loading dye to each sample. We then loaded 10 μL of each sample to the wells in the gel and ran the gel at 150V for 30 minutes. After rinsing the gel, we added the SYBR safe stain, covered it with aluminum foil and placed it in the shaker for 30 minutes. Finally, we rinsed the gel and observed the fluorescence of bands using the fluorescence gel imager at 488 nm excitation and 522 nm emission using manual exposure of 1.25 seconds. [1]

3.0 Results

In this section, images of our PAGE gel and an example gel are provided. The corresponding concentrations of Thrombin and aptamer in each sample, along with the amount of volumes used in preparation and calculated Thrombin/aptamer ratios are presented in *Tables 1 and 2*.

In Figure 1, we observe bands forming across wells. We observe the "F" band, which corresponds to free (unbound) DNA-based TBA aptamer in samples TBA Control and from samples T-0.125 to T-8. This band is not visible in T-16, T-32 and NA. We observe C1 and C2 bands forming clearly in samples T-4 through T-32. These bands are briefly visible in wells loaded with T–0.5, T-1 and T-2. We observe the C3 band only in T-16 and T-32. We also observe C1 and C2 bands forming in the "empty" well which is labelled as NA (Well 12).

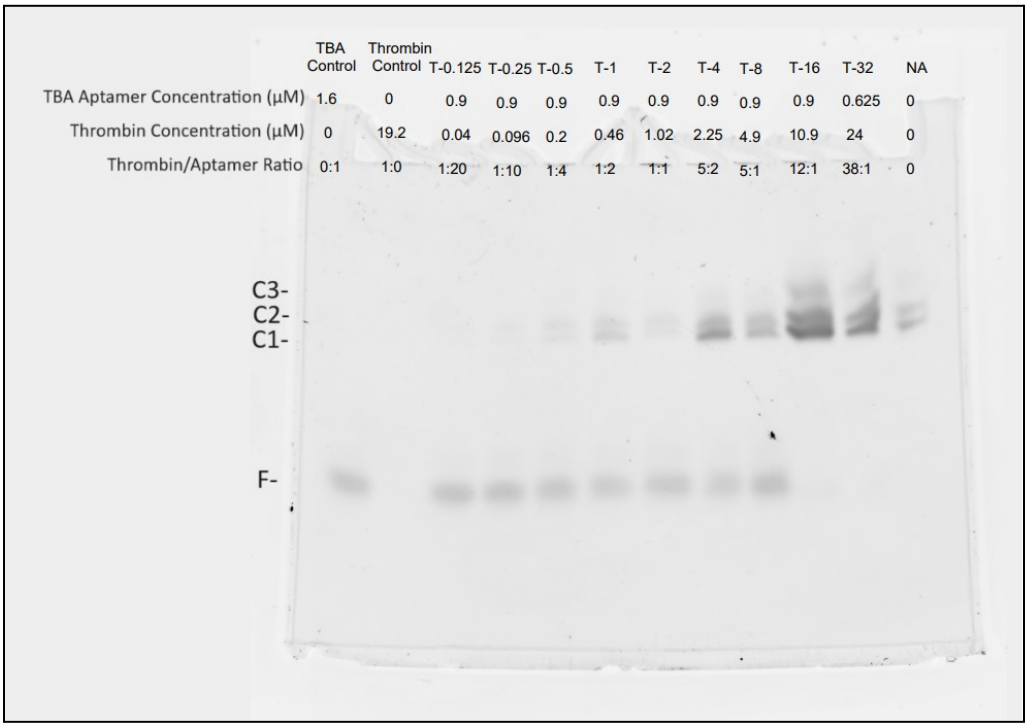

Figure 1: Our PAGE Gel with Thrombin-aptamer complexes with corresponding Thrombin and aptamer concentrations in µM and Thrombin/aptamer ratios.

All except Thrombin Control contain equal amounts of TBA aptamer (10 µM). All except TBA Control contain Thrombin. Since the Thrombin was prepared in serial dilutions, each sample contains a different concentration. The volumetric amounts used when preparing the samples are presented in *Table 1* below, along with the exact concentrations in each sample and their respective Thrombin/Aptamer ratio. All samples in the PAGE Gel in Figure 1 contained equal amounts of aptamer binding reaction buffer (100 mM KCl). Electrophoresis was carried out at room temperature in a 4-15% precast 12-well polyacrylamide gel run at 150V for 30 minutes. Symbols in Figure 1 correspond to: F, Free DNA-based TBA aptamer; C1-3: Thrombin-aptamer complexes.

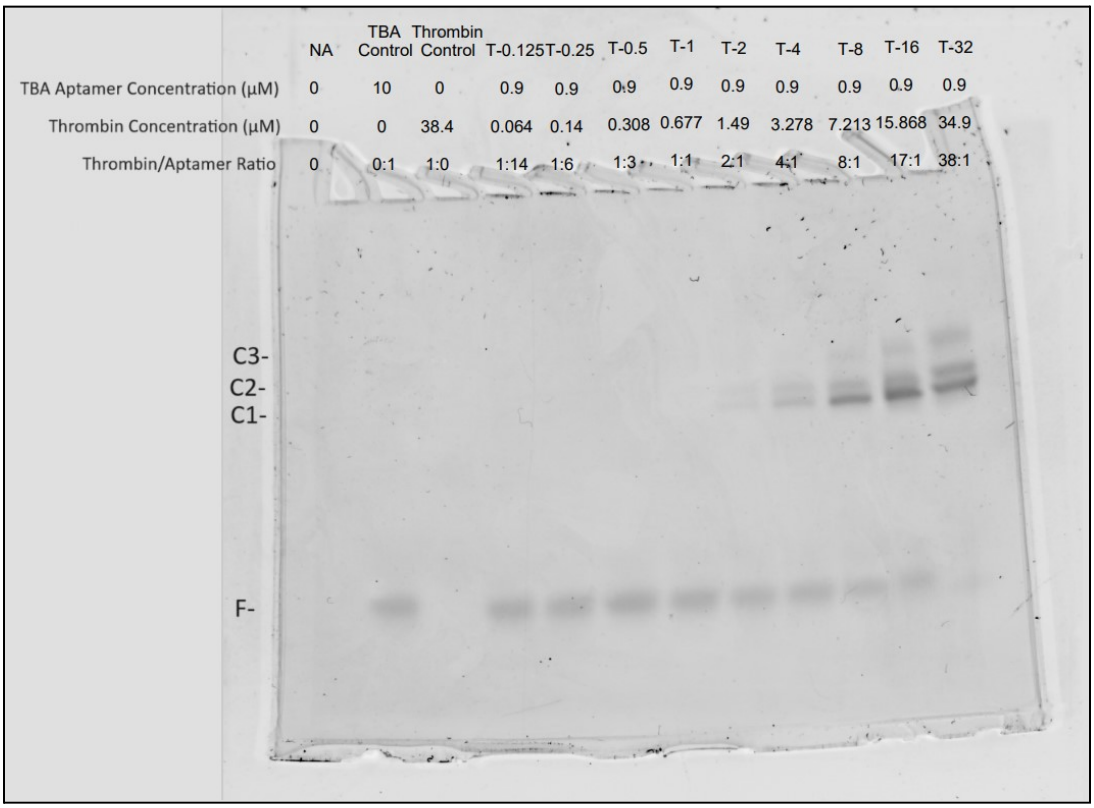

Figure 2: Example PAGE Gel with Thrombin-aptamer complexes with corresponding Thrombin and aptamer concentrations in µM and Thrombin/aptamer ratios.

All except Thrombin Control contain equal amounts of TBA aptamer (10 µM). All except TBA Control contain Thrombin. Since the Thrombin was prepared in serial dilutions, each sample contains a different concentration. The volumetric amounts used when preparing the samples are presented in *Table 2* below. All samples except T-32 and the control samples in the PAGE Gel in *Figure 2* contain equal amounts of aptamer binding reaction buffer (100 mM KCl). Electrophoresis was carried out at room temperature in a 4-15% precast 12-well polyacrylamide gel run at 150V for 30 minutes. Symbols in Figure 1 correspond to: F, Free DNA-based TBA aptamer; C1-3: Thrombin-aptamer complexes.

Table 1: Corresponding calculated concentrations of TBA aptamer and Thrombin in each sample with their respective volumetric amounts used in preparation of samples in our PAGE gel in Figure 1. Thrombin/Aptamer Ratio represents the Thrombin concentration divided by the TBA aptamer concentration in each sample. where the concentrations are rounded to the nearest integers for simplicity.

	Volume (µL)				Concentration (µM)		
Sample	**Aptamer**	**Thrombin**	**Aptamer Binding Reaction Buffer (BRB)**	**Total**	**Thrombin**	**Aptamer**	**Rounded Thrombin/Aptamer Ratio**
Thrombin-32	2 µL	20 µL	10 µL	32 µL	24 µM	0.625	38:1
Thrombin-16	2 µL	10 µL	10 µL	22 µL	10.9 µM	0.9 µM	12:1
Thrombin-8	2 µL	10 µL	10 µL	22 µL	4.9587 µM	0.9 µM	5:1
Thrombin-4	2 µL	10 µL	10 µL	22 µL	2.254 µM	0.9 µM	5:2
Thrombin-2	2 µL	10 µL	10 µL	22 µL	1.0245 µM	0.9 µM	1:1
Thrombin-1	2 µL	10 µL	10 µL	22 µL	0.4657 µM	0.9 µM	1:2
Thrombin-0.5	2 µL	10 µL	10 µL	22 µL	0.2117 µM	0.9 µM	1:4
Thrombin-0.25	2 µL	10 µL	10 µL	22 µL	0.0962 µM	0.9 µM	1:10
Thrombin -0.125	2 µL	10 µL	10 µL	22 µL	0.0437 µM	0.9 µM	1:20
Thrombin Control	0	10 µL	10 µL	20 µL	19.2 µM	0	1:0
TBA Control	2 µL	0	10 µL	12 µL	0	1.6 µM	0:1

Table 2: Corresponding calculated concentrations of TBA aptamer and Thrombin in each sample with their respective volumetric amounts used in preparation for the Example PAGE Gel in Figure 2. Thrombin/Aptamer Ratio represents the Thrombin concentration divided by the TBA aptamer concentration in each sample. where the concentrations are rounded to the nearest integers for simplicity.

	Volume (µL)				Concentration (µM)		
Sample	**Aptamer**	**Thrombin**	**Aptamer Binding Reaction Buffer (BRB)**	**Total**	**Thrombin**	**Aptamer**	**Rounded Thrombin/Aptamer Ratio**
Thrombin-32	2 µL	20 µL	0	22 µL	34.9 µM	0.9 µM	38:1
Thrombin-16	2 µL	10 µL	10 µL	22 µL	15.868 µM	0.9 µM	17:1
Thrombin-8	2 µL	10 µL	10 µL	22 µL	7.213 µM	0.9 µM	8:1
Thrombin-4	2 µL	10 µL	10 µL	22 µL	3.278 µM	0.9 µM	4:1
Thrombin-2	2 µL	10 µL	10 µL	22 µL	1.49 µM	0.9 µM	2:1
Thrombin-1	2 µL	10 µL	10 µL	22 µL	0.677 µM	0.9 µM	1:1
Thrombin-0.5	2 µL	10 µL	10 µL	22 µL	0.308 µM	0.9 µM	1:3
Thrombin-0.25	2 µL	10 µL	10 µL	22 µL	0.14 µM	0.9 µM	1:6
Thrombin -0.125	2 µL	10 µL	10 µL	22 µL	0.064 µM	0.9 µM	1:14
Thrombin Control	0	10 µL	0	10 µL	38.4 µM	0	1:0
TBA Control	2 µL	0	0	2 µL	0	10 µM	0:1

4.0 Discussion

In both *Figures 1 and 2,* we observe 3 bands: C1, C2 and C3. These bands are formed due to our DNA-based aptamer sample having DNA segments in varying sizes [3]. The smaller DNA molecules move further in the gel, creating the C1 band. In the interpretation of results we will mainly consider this band, since it is visually more clear.

As mentioned in the Results section, in *Figure 1*, we only observe the F band in TBA Control sample, since there is no Thrombin that aptamer can bind to in this sample. For relatively low concentrations of thrombin, in samples T-0.125 through T-8, we continue to observe this band. As the F band gets lighter, it indicates that there is less and less free aptamer in the sample with increasing thrombin concentration. As the F band ceases, we observe the shifted C1 band forming clearly starting from sample T-4, indicating the formation of thrombin-aptamer complexes that are larger in size. Finally we observe the C1 band clearly forming for the samples T-16 and T-32, with nothing in the F band. Since there is no free DNA-based aptamer in the solution, we don't observe an F band. This indicates that all TBA aptamer in the sample is bound to the Thrombin protein, creating a darker band as there are more complexes. We can observe that the C1 band in T-32 is quite similar compared to T-16, indicating that for larger Thrombin concentrations, there is no more aptamer remaining for binding.

The results from our gel, aligns with our initial hypothesis of higher protein:nucleic acid ratios creating more complexes with darker, shifted bands in our gel until there is no longer free aptamer present in the solution. However in our gel, we also observe some unexpected results in comparison to the example gel in *Figure 2.* As mentioned in the Results section, we observe C1 and C2 band formation in the supposedly empty well (well 12, labeled NA). This is likely caused by a spill-over during the loading of the samples. We also observe these bands are forming in T-0.5, T-1 and T-2 samples, which could result from defects when transferring the liquids in preparation of the samples, which may have resulted in slightly higher Thrombin concentrations compared to the given example. We observe that the T-4 sample demonstrates darker bands than the T-8, which is also potentially due to a defect in transferring volumes. Although it doesn't impact our ability to compare our gel with the example gel, I'd also like to point out the difference of volumes of BRB used in the preparation of our samples, in comparison to the provided example, which resulted in different concentrations and ratios.

In this experiment, we used EMSA rather than SDS-PAGE for this assay, because EMSA separates molecules based on their charge in addition to their size, which is useful when determining the binding stoichiometry since proteins interact with DNA through electrostatic interactions [4]. ChIP, DNA pull-down, microplate capture and detection and reporter assays can also be used for our purpose [4]. Especially DNA pull-down assays would allow us to learn more about the protein-DNA interaction.

5.0 References

1. Poon, W; Fiddes, L; Hoang, T.G.; Stordy, B.; Chou, L., 2023-2024 BME440H Lab 2: Protein Binding & Native PAGE EMSA, Lab II, University of Toronto, Department of Biomedical Engineering, 2023.

2. Hellman, L. M.; Fried, M. G. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2757439/ (Accessed Oct 13, 2023).

3. Gel electrophoresis https://www.hudsonalpha.org/gel-electrophoresis-3/#:~:text=This%20makes%20sense%20because%20when,because%20of%20the%20uncut%20fragments. (Accessed Oct 13, 2023).

4. Methods for detecting protein-DNA interactions: Thermo Fisher Scientific - US https://www.thermofisher.com/ca/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/methods-detecting-protein-dna-interactions.html (Accessed Oct 13, 2023).