Plasma Protein Binding of Oligonucleotides Using Rapid Agarose Gel Electrophoretic Mobility Shift Assay

Jie Wang, Mengying Li, Xiangling Wang, Yi Tao, Liang Shen, Genfu Chen
DMPK Service Department, WuXi AppTec, 31 Yiwei Road, Waigaoqiao Free Trade Zone, Shanghai, P.R. China, 200131

药明康德 WuXi AppTec

Poster#: P105

Abstract

50KD-Ultrafiltration (UF) method and electrophoretic mobility shift assay (EMSA)-based plasma protein binding (PPB) approaches have been reported previously for oligonucleotides (oligos) in the literatures[1]. However, there are challenges for both methods. In order to establish the best practices for PPB of oligos, we developed a rapid agarose gel EMSA with Invitrogen E-Gel Power Snap electrophoresis device for assessing the PPB of 4 phosphorothioate antisense oligonucleotide (PS-ASOs) and 4 GalNac-siRNAs, and the data were compared with the previously reported data in literatures. Results showed that PPB values of the 4 PS-ASOs were generally in good agreement qualitatively with the reported values. The PPB values of the 4 siRNAs obtained by EMSA method were different from reported data.

Objectives

To develop a rapid agarose gel EMSA method for PPB measurement of oligos.

Methods

Items	Name	Vender
Compounds	ASOs / siRNAs	MedChemExpress / WuXi STA TIDES team
Instrument	E-Gel™ Power Snap Electrophoresis Device	Thermo Fisher Scientific
Matrix	Human Plasma	BioIVT

Incubation
- The ASOs or siRNAs were diluted by nuclease-free water (for ASOs) or 1×Tris-EDTA (TE) Buffer, pH8.0 (for siRNAs).
- Human plasma were diluted with nuclease-free water (for ASOs) or TE Buffer (for siRNAs) to get 5% or 10% diluted plasma.
- Oligos were mixed with the diluted plasma and incubated at 37 °C for 30 minutes.

Loading Samples
- Agarose gels (2% or 4%) containing SYBR Gold as the stain were used.
- Samples of 20 µL were loaded into each well of agarose gel.
- Electrophoresis for 3 to 8 minutes on ice.

Analysis
- Photos of the gels were taken using the E-Gel™ Power Snap Camera.
- The software ImageJ 1.53k was used to calculate the integrated density of bands on the gel.
- Lines of best fit were determined between concentrations and integrated densities.

Calculation
- Apparent fraction unbound in loading plasma (which is diluted plasma, diluted f_u) and undiluted f_u (in neat plasma extrapolated using the correction factor equation) are determined by the following equations, where D is the dilution factor.
- %Unbound = 100 × f_u

$$\text{Diluted } f_u = \frac{\text{concentration in the free band}}{\text{concentration in the neat band}}$$

$$\text{Undiluted } f_u = \frac{1/D}{((1/\text{Diluted fu})-1)+1/D}$$

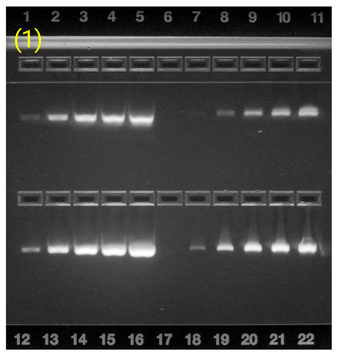
- Neat band means the band of oligos in water or buffer. Free band means the band of unbound oligos in plasma, which migrates more quickly than protein bound-oligos.

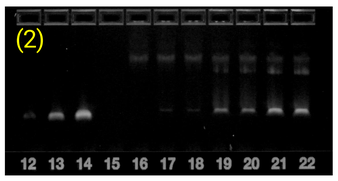
Results

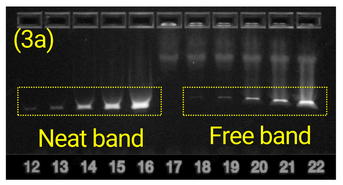
Table 1. PPB values of the 4 ASOs (5% diluted plasma, n=3).

ASOs	Concentration (µM)	%Undiluted Unbound in neat plasma Mean ± SD	Apparent %Unbound in loading plasma Mean ± SD	Literature %Unbound Values[2,3]
Mipomersen	1	1.2 ± 0.6	18.8 ± 7.8	4.18 ~ 5.65 in human plasma and 0.93 ~ 1.63 in 600 µM Albumin from 1 ~ 10 µM
	5	1.3 ± 0.1	20.7 ± 1.7	
	10	1.6 ± 0.7	24.0 ± 9.3	
Nusinersen	1	1.1 ± 0.4	17.6 ± 5.6	3.9 ~ 5.9 in human plasma from 0.7 ~ 14 µM
	5	4.6 ± 0.5	49.1 ± 2.7	
	10	5.7 ± 1.1	54.4 ± 5.1	
Volanesorsen	1	0.2 ± 0.2	3.6 ± 4.0	0.76 ~ 1.99 in human plasma from 0.7 ~ 21 µM
	5	0.3 ± 0.2	5.3 ± 4.4	
	10	2.6 ± 0.4	34.2 ± 4.3	
Inotersen	1	3.1 ± 1.0	38.3 ± 8.4	2.2 ~ 5.6 in human plasma from 0.7 ~ 20 µM
	5	0.9 ± 0.1	15.9 ± 1.8	
	10	0.6 ± 0.2	11.3 ± 2.6	

Table 1 showed that the 4 PS-ASOs by EMSA were highly bound in human plasma, which is consistent with the literature values in a qualitative manner. Besides, they were also consistent with in house data by UF method (data not shown). In other words, the PPB classifications of these ASOs were in good agreement with the other methods.







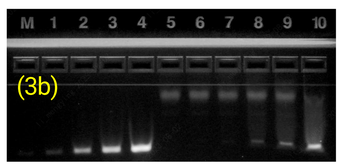


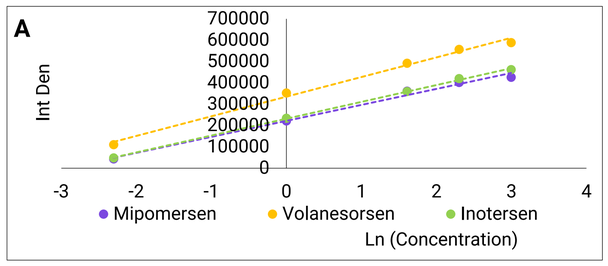
Figure 1. 0.1, 1, 5, 10, 20 µM of ASOs in water. 2% agarose gel was run for 3 min.
1 ~ 5: Mipomersen; 7 ~ 11: Nusinersen; 12 ~ 16: Volanesorsen; 18 ~ 22: Inotersen.

Figure 2. Nusinersen in water and 5% human plasma. 2% agarose gel was run for 3 min.
12 ~ 15: 1, 5, 10, 0 µM of neat Nusinersen; 16 ~ 22: 0, 1, 1, 5, 5, 10, 10 µM of Nusinersen in diluted plasma.

Figure 3. Inclisiran in TE Buffer and 10% human plasma. Agarose gels were run for 3 min in 2% gel (figure 3a) and 8 min in 4% agarose gel (figure 3b). 12 ~ 16 & M ~ 4 : 0.5, 1, 5, 10, 25 µM of neat Inclisiran in TE buffer; 17 ~ 22 & 5 ~ 10: 0, 0.5, 1, 5, 10, 25 µM of Inclisiran in diluted plasma.

Table 2. The linear equations of 4 ASOs and 4 siRNAs. For Mipomersen, Volanesorsen and Inotersen, linear curves are obtained by plotting the integrated densities against the natural log (Ln)-transformed concentrations (curves were shown in Figure 4 A). For Nusinersen and the 4 siRNAs, linear curves are obtained by plotting ln-transformed integrated densities against ln-transformed concentrations (Figure 4 B).

ASOs	Linear Equation	R^2	siRNAs	Linear Equation	R^2
Mipomersen	y = 74932x + 221738	0.9917	Inclisiran	y = 0.8462x + 10.218	0.9849
Nusinersen	y = 0.7375x + 10.642	0.9902	Lumasiran	y = 0.5652x + 10.792	0.9920
Volanesorsen	y = 91959x + 335085	0.9926	Givosiran	y = 0.7285x + 10.129	0.9813
Inotersen	y = 78938x + 231781	0.9992	Vutrisiran	y = 0.9848x + 9.8121	0.9718



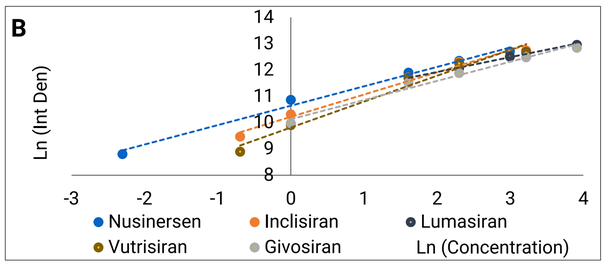


Figure 4. Lines of best fit of 3 ASOs (A) and the other oligos (B).

Table 3. The PPB of siRNAs in human plasma (10% diluted).

siRNAs	Concentration (µg/mL)	%Undiluted Unbound Mean ± SD in 2% Agarose Gel	Apparent %Unbound Mean ± SD in 2% Agarose Gel	%Undiluted Unbound in 4% Agarose Gel (mean of 2 experiments)	Apparent %Unbound Mean in 4% Agarose Gel (mean of 2 experiments)
Inclisiran	0.5	3.1 ± 0.7 (N=4)	24.0 ± 4.4	NA	NA
	1	4.8 ± 3.0 (N=4)	31.9 ± 12.4	0.7 (0.9, 0.5)	6.2 (7.9, 4.5)
	5	5.5 ± 3.2 (N=4)	34.9 ± 12.3	1.9 (2.9, 0.9)	15.7 (22.8, 8.6)
	10	6.4 ± 1.4 (N=4)	40.3 ± 5.2	2.6 (3.7, 1.6)	20.9 (27.8, 14.0)
	25	5.8 ± 0.2 (N=4)	37.9 ± 1.0	3.1 (3.7, 2.5)	23.9 (27.5, 20.3)
Lumasiran	5	0.3 ± 0.3 (N=7)	2.9 ± 2.3	0.4 (0.4, 0.4)	4.1 (4.3, 3.8)
	10	0.6 ± 0.2 (N=7)	5.8 ± 2.2	1.6 (2.5, 0.7)	13.7 (20.5, 6.9)
	20	1.2 ± 0.7 (N=7)	10.9 ± 5.5	2.7 (3.9, 1.5)	21.1 (28.7, 13.5)
	25	1.7 ± 0.8 (N=7)	14.3 ± 6.2	4.8 (7.9, 1.7)	30.5 (46.2, 14.9)
	50	3.5 ± 2.1 (N=7)	25.0 ± 11.8	8.2 (12.3, 4.1)	44.3 (58.4, 30.2)
Givosiran	0.5	2.0 ± 0.2 (N=6)	19.2 ± 5.6	NA	NA
	1	2.3 ± 1.2 (N=6)	20.3 ± 7.6	0.6 (0.6, 0.6)	5.5 (5.4, 5.5)
	5	3.0 ± 3.4 (N=6)	21.7 ± 16.2	0.9 (0.6, 1.2)	8.3 (6.0, 10.5)
	10	2.8 ± 2.8 (N=6)	21.6 ± 14.5	2.2 (2.4, 2.0)	18.5 (19.9, 17.2)
	25	3.0 ± 2.7 (N=5)	21.7 ± 14.9	2.9 (3.2, 2.5)	22.9 (25.1, 20.7)
Vutrisiran	1	NA	NA	NA	NA
	5	1.1 ± 0.3 (N=3)	9.7 ± 2.7	NA	NA
	10	3.4 ± 0.1 (N=3)	25.7 ± 0.7	1.2 (1.3, 1.1)	10.8 (12.0, 9.6)
	25	4.1 ± 0.7 (N=3)	29.7 ± 3.7	2.4 (2.9, 2.0)	19.9 (23.0, 16.7)
	50	5.5 ± 0.0 (N=3)	37.0 ± 0.1	5.3 (6.9, 3.7)	35.1 (42.5, 27.8)

- NA means low sensitivity to detect the protein-free samples. N represents the number of independent experiments.

1. Larger variations between experiments were observed for these siRNAs.
2. The 4 siRNAs were highly bound in human plasma, in both 2% and 4% gels.
3. The in house unbound values (by EMSA) were lower than the reported data at clinical relevant concentrations: 13% fraction unbound for Inclisiran at 0.5 µg/mL, 15% for Lumasiran at 1 µg/mL, 8% for Givosiran at 1 µg/mL, 18% for Vutrisiran at 1 µg/mL.
4. The in house unbound values (by EMSA) were inconsistent with in house UF method at 1 µg/mL: 11.8% for Inclisiran, 13.0% for Lumasiran, 10.1% for Givosiran, 17.6% for Vutrisiran.
5. Reasons for the inconsistence for siRNA PPB were unknown, possibly at least in part due to the limitations of the EMSA technique, which requires separation of bound protein-nucleic acid complexes from free nucleic acids in an electric field. Other possible reasons are the effects of diluting buffer compositions and pH, which should be further investigated.

Conclusion

1. A rapid agarose gel EMSA method was developed for determining PPB of oligos. Our method used very short run times (a few minutes), which should reduce opportunities for dissociation of bound complexes, an important concern for EMSA.
2. The reliability of this method was validated by qualitatively comparing data of the 4 PS-ASOs with literatures as well as the in house UF data. Different PPB values were found for the four FDA-approved GalNac-siRNAs in contrast to the reported data, or data from the in house UF method.
3. We recommended that EMSA should be used as a qualitative technique for determining PPB in plasma during screening phases. Alternative method, such as ultrafiltration is recommended in the late stages and a head-to-head data comparison with reference compound is encouraged.

References

[1] Considerations and recommendations for assessment of plasma protein binding and drug-drug interactions for siRNA therapeutics. Nucleic Acids Res. 2022 Jun 24;50(11):6020-6037.
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Contact