Reaction condition optimization to minimize hydrolysate formation in a maleimide-NHS ester-oligonucleotide conjugation reaction

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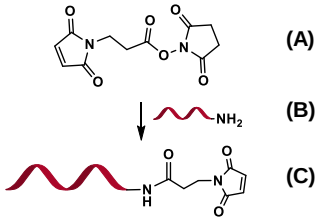
Abstract

Maleimide chemistry is unique in the tool-kit of bioconjugation because of its ease of synthesis and remarkable reactivity. 3-(Maleimido)propionic acid N-hydroxysuccinimide ester (A) reacts with the amine group of amino-modified DNA (B), in a basic environment leads to the incorporation of maleimide into oligonucleotide strand (C), Fig. 1. The newly introduced maleimide is susceptible to base catalyzed hydrolysis in aqueous environments, producing hydrolysate (D) – an impurity. By altering three key reaction parameters - temperature, time, and pH - we set out to optimize the reaction conditions to slow down the formation of hydrolysate during conjugation reaction. We employed design of experiments (DOE) with the JMP software to minimize the number of experiments required for this study.



Oligo conjugated with maleimide (DNA+maleimide)

Figure 1. Conjugation reaction

Background

The conjugation reaction requires basic condition. However, in aqueous condition the maleimide rings is opened by hydrolysis forming hydrolysate **(D).**

Hydrolysis rate increases with high pH.

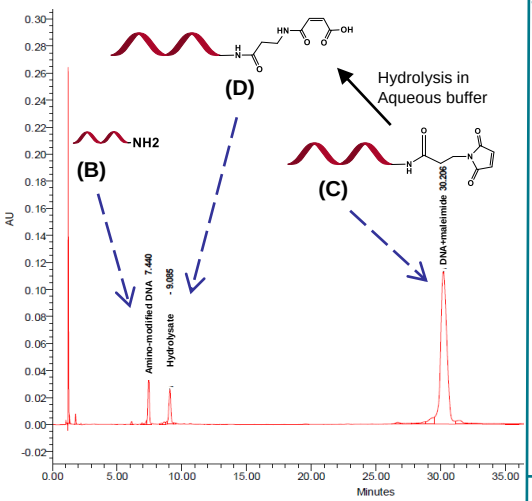


Figure 2. Example of LE IPRP chromatogram displaying peaks representing (B), (C), and (D). Solution of (A) in DMSO and aqueous solution of (B) were mixed at 20 ± 5 °C for 60 min.

Reactions parameters for DoE

Design of Experiment (DOE) with JMP was used to assess process parameters deemed potentially influential for successful conjugation of (A) with (B) to (C) while keeping hydrolysate (D) low.

For conjugation reaction, JMP used to generate a weighted D-optimal assessment to evaluate the following parameters: pH, temperature, and time.

3 Factor design were utilized with the following levels for each Discrete numeric factor (3 inputs)

- pH = 4 (5.5, 6, 6.5, 7.2)
- Temp = 3 (20°C, 25°C, 35°C)
- Time = 3 (30 min, 60 min, 120 min)

DoE design, and Raw Results

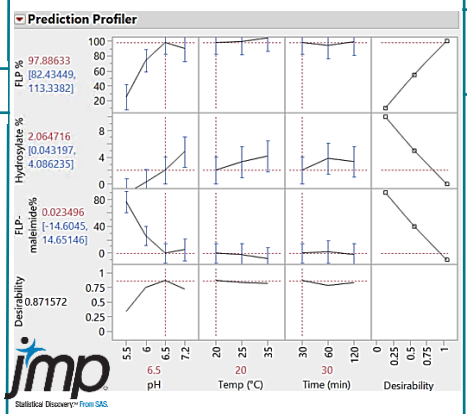
3 outputs of the design

- %DNA+Maleimide [%(C)], desirability maximum
- %Hydrolysate [%(D)], desirability minimum
- %Amino-modified-DNA [%(B)], desirability minimum

Table 1. 12 experiments proposed by JMP (B1 - B12)

Sample Name	Rxn Condition (Input)			Normalized Value (Output)		
	Rxn pH	Temp, °C	Time, min	%Maleimide-DNA (%C)	%Hydrolysate (%D)	%Amino-modified-DNA (%B)
B3	5.5	20	60	17.9	0.5	81.6
B11	6		120	77.7	2.4	19.9
B6	6.5		30	92.0	2.3	5.6
B12	7.2		30	**96.5**	3.5	0.0
B9	5.5	25	30	20.1	0.5	79.4
B8	6		60	83.0	2.4	14.6
B5	6.5		120	**95.0**	4.5	0.6
B7	7.2		120	92.5	7.5	0.0
B1	5.5	35	120	35.0	0.9	64.1
B10	6		30	85.9	2.7	11.5
B4	6.5		60	94.1	5.4	0.4
B2	7.2		60	90.2	9.8	0.0

Predicted optimal conditions



- pH 6.5, temperature **20 °C**, time 30 min

Confirmation Experiments

Table 2. Optimal pH and time was investigated at 20 °C

Sample Name	Rxn Condition		Normalized Value		
	Rxn pH	Time, min	%Maleimide-DNA (%C)	%Hydrolysate (%D)	%Amino-modified-DNA (%B)
S1	6.5	30	87.2	2.1	10.7
S2		60	91.4	2.6	6.0
S3		120	94.1	3.5	2.4
S4	6.9	30	**96.7**	**2.4**	**0.8**
S5		60	**96.4**	**3.3**	**0.3**
S6		120	95.5	4.5	0.0
S7	7.2	30	96.5	3.5	0.0

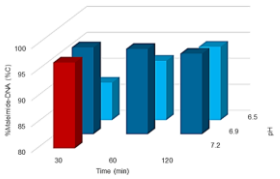


Figure 3. DNA+Maleimide profile

Complete or near-complete conversion observed while hydrolysis rate limited to ~ 2-3% (S3 - S7).

Manufacturing optimal conditions for 60 mmol:, pH 6.9, 60 min, 20 °C.

Conclusion and Outlook

For the conjugation reaction, optimal reaction conditions were designed using DOE/JMP

Utilization of statistical software have potential to decrease time spent to perform experiments while evaluating a wide range of parameters.

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