

Submission ID #: 62597

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Project Page Link: <https://www.jove.com/account/file-uploader?src=19092133>

Title: Activation and Conjugation of Soluble Polysaccharides Using 1-Cyano-4-Dimethylaminopyridine Tetrafluoroborate (CDAP)

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Interview statements: Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one.**

☒ Interviewees wear masks until videographer steps away (≥ 6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 15

Number of Shots: 36

Introduction

1. Introductory Interview Statements

REQUIRED:

- 1.1. **Andrew Lees:** Polysaccharides are poorly immunogenic unless linked to protein and need to be activated for derivatization or conjugation to proteins. In this method, CDAP was used as a cyanylating reagent, which activates polysaccharide hydroxyls to be derivatized or linked to proteins for use in vaccines and diagnostic reagents.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.2 for 'cyanylating reagent'*

- 1.2. **Andrew Lees:** CDAP is simpler to use, more efficient, and can be used at a lower pH. It is a better activating reagent and has largely replaced cyanogen bromide as a polysaccharide cyanylating reagent. *Suggested B-roll: 2.2.3 for 'lower pH'*

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

- 1.3. **Andrew Lees:** CDAP can be used with most polysaccharides, unlike reductive amination with periodate, which requires vicinol hydroxyls. The chemistry can be used to both, directly and indirectly conjugate proteins and make diagnostic reagents.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

- 1.4. **Andrew Lees:** The CDAP activation reactions are very rapid and difficult to control. The protocol provided here makes the chemistry far easier to perform and more reproducible.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Polysaccharide Activation and Hydrazide Functionalization

- 2.1. Before starting the experiment, adjust the pH of the polysaccharide solution to 9 [1] by adding 200 microliters of dimethylaminopyridine stock solution in a dropwise manner while stirring [2-TXT]. Keep the reaction chilled in an ice-water bath for the entire process of the activation [3]. *Videographer: This step is important!*

2.1.1. WIDE: Talent placing reaction beaker in an ice-water bath and placing the pH probe into the polysaccharide solution.

2.1.2. Talent adding dimethylaminopyridine stock solution to the polysaccharide under stirring and adjusting pH if necessary. **TEXT: Avoid exceeding target pH by more than 0.1 pH unit**

2.1.3. Reaction beaker on an ice-water bath

Videographer's NOTE: 2.1.2 and 2.1.3 were filmed in one shot.

- 2.2. Proceed with Cyano-Dimethyl-aminopyridine Tetrafluoroborate or CDAP activation by transferring 100 microliters of CDAP to the polysaccharide solution while stirring [1-TXT]. Start the timer and monitor the pH change during the entire activation for 15 minutes [2]. Maintain the reaction at the target pH by promptly adding 10-microliter increments of 0.1 molar sodium hydroxide [3]. *Videographer: This step is important!*

2.2.1. Talent adding CDAP to the polysaccharide solution with stirring. **TEXT: CDAP: 1-cyano-4-dimethylaminopyridine tetrafluoroborate**

2.2.2. Talent starting the timer for 15 minutes. Reaction beaker with the pH probe in the view.

Videographer's NOTE: 2.2.1 and 2.2.2 were filmed simultaneously.

2.2.3. Reading on the pH meter and talent adding sodium hydroxide to the reaction.

Videographer's NOTE: 2.2.3 was not done here. Done at the beginning of 2.2.1.

- 2.3. When the optimal activation time is reached, add 2 milliliters of 0.5 molar adipic dihydrazide all at once to the activated polysaccharide [1]. Check that the pH is in the required range for hydrazide functionalization [2-TXT]. *Videographer: This step is important!*

2.3.1. Talent adding adipic dihydrazide to the activated polysaccharide under stirring.

- 2.3.2. Readings on the pH meter. **TEXT: pH 8–9 for adipic dihydrazide**
- 2.4. After continuous stirring of the reaction mixture for at least 1 hour, transfer the reaction mixture to 4 degrees Celsius [1-TXT].
 - 2.4.1. Talent transferring the reaction mixture to the fridge. **TEXT: 0–20 °C is acceptable**
- 2.5. To reduce ADH concentration from the product, dialyze the crude derivatized polysaccharide solution using the dialysis device [1]. Recover the derivatized polysaccharide from dialysis [2] and determine the concentration of the polysaccharides and hydrazide to calculate the hydrazide to polysaccharide ratio [3-TXT]. *Videographer: This step is important!*
 - 2.5.1. Dialysis of the polysaccharide solution.
 - 2.5.2. Talent collecting the derivatized polysaccharide from dialysis.
 - 2.5.3. Talent entering readings on a worksheet. **TEXT: Refer to text if dialyzed polysaccharide needs to be concentrated**

3. Analysis of Hydrazide-Derivatized Polysaccharide

- 3.1. Prepare 1 milligram per milliliter unmodified polysaccharide solution to be used as the standard [1]. Pre-heat the heating block to 140 degrees Celsius for a minimum of 1 hour to achieve a stable, uniform temperature. Use a protective pad underneath and surrounding the heating block in case of acid spills [2].
 - 3.1.1. WIDE: Standard polysaccharide solution in a labeled tube.
 - 3.1.2. Talent turning the heating block ON.
Videographer's NOTE: 3.1.1 and 3.1.2 were done at the beginning of 3.7.1
- 3.2. Add varying volumes of 1 milligram per milliliter carbohydrate standard to the labeled 13 by 100 borosilicate test tubes in triplicates [1-TXT] and make up the volume to 100 microliters with water to achieve the desired standard concentrations [2].
 - 3.2.1. Talent adding carbohydrate standard to the labeled borosilicate test tubes.
TEXT: 0, 2.5, 5, 7.5, 10 µg carbohydrate standard **Videographer's NOTE: Use 3.2.1 take 2**
 - 3.2.2. Talent adding water to the test tubes.

Videographer's NOTE: 3.2.2 was filmed at the beginning of 3.2.1. Use 3.2.1 take 1.

- 3.3. Similarly, set up sample assays by adding a volume containing 5 micrograms of the derivatized polysaccharide to three sample tubes [1]. Add 100 microliters of freshly prepared 6 milligrams per milliliter resorcinol to each tube [2].

3.3.1. Talent adding derivatized polysaccharide and water to the sample tubes.

3.3.2. Talent adding resorcinol to each tube.

Videographer's NOTE: 3.3.1 and 3.3.2 were filmed in one shot.

- 3.4. Using a repeat pipettor, add 300 microliters of the prepared 75% sulfuric acid to each tube [1-TXT]. Vortex the tubes vigorously, pointing the tube away [2], then place all the tubes in a heater block at a steady pace in sequential order [3] and immediately set the timer for 3 minutes [4].

3.4.1. Talent adding sulfuric acid to the tube. **TEXT: Refer to text for preparation of 75% sulfuric acid**

3.4.2. Talent vortexing the tubes.

Videographer's NOTE: 3.4.1 and 3.4.2 were filmed in one shot.

3.4.3. Talent placing all the tubes in a heater block.

3.4.4. Talent setting the timer for 3 minutes. The tubes in a heater block are visible in the frame.

- 3.5. At 3 minutes, remove the tubes in the same order [1] and place them directly in a rack in an ice water bath [2]. Remove the ice-cold tubes to allow them to equilibrate to room temperature for 5 minutes [3].

3.5.1. Talent removing tubes from the block. Videographer's NOTE at 3.5.1: Cut before last 2 samples removed.

3.5.2. Talent placing tubes in an ice water bath.

3.5.3. The tubes in an appropriate container, sitting on the lab bench. Timer Visible in the frame.

- 3.6. Read the absorbance of all the tubes on a UV/VIS spectrophotometer at 430 nanometers using a 10-millimeter pathlength cuvette [1].

3.6.1. Talent recording the absorbance.

- 3.7. To set up assay reactions, sort and arrange the labeled standard tubes in the tube rack in order of increasing concentration [1]. Using a calibrated micropipette, add 100 microliters of the standards to each corresponding tube [2]. For the zero standard, use 100 microliters of the sample buffer [3].

3.7.1. The labeled standard tubes in a tube rack.

3.7.2. Talent adding the standards to each tube.

3.7.3. Talent adding the sample buffer into labeled zero standard tubes.

Videographer's NOTE for 3.7.3: Already in tubes.

- 3.8. Similarly, prepare and arrange the sample tubes [1]. Then, using a calibrated 1000-microliter micropipette, add 875 microliters of Assay buffer to all assay tubes [2].

3.8.1. Labeled sample tubes in a tube rack, sitting on the lab bench.

3.8.2. Talent adding Assay buffer to all assay tubes.

- 3.9. To start the assay reaction, add 25 microliters of 1% trinitrobenzene sulfonic acid to each assay tube in the pre-determined order within 5 minutes [1-TXT]. Vortex all assay tubes for 2 seconds at high speed, making sure that the liquid swirls to a height of half an inch from tube opening [2].

3.9.1. Talent adding TNBS to labeled assay tubes. **TEXT: Trinitrobenzene sulfonic acid: TNBS**

3.9.2. Talent vortexing assay tubes. *Videographer: Take multiple shots to be used again in 3.10.3*

- 3.10. Record the assay start time and set the timer to 2 hours [1]. Then, place the assay tube rack in the dark at room temperature for 2 hours [2]. When the time is over, vortex all the tubes and proceed to data collection [3].

3.10.1. Talent setting the timer.

3.10.2. Talent placing place the assay tube rack in the dark. **Videographer's NOTE: 3.10.2 was filmed at the end of 3.9.2.**

3.10.3. *Use 3.9.2.*

Results

4. Results: Evaluation of Dextran, A Polysaccharide After CDAP Activation

- 4.1. The ADH-dextran was assayed for dextran using the resorcinol-sulfuric acid assay [1-TXT]. A typical standard curve using glucose as the sugar standard was plotted [2].
 - 4.1.1. LAB MEDIA: Figure 4 B. **TEXT: ADH: Adipic dihydrazide**
 - 4.1.2. LAB MEDIA: Figure 4 B. *Video Editor: Graph in the left panel. Emphasize the y-axis title.*
- 4.2. The hydrazide content in derivatized polysaccharide or dextran was determined using the TNBS assay [1].
 - 4.2.1. LAB MEDIA: Figure 4 A.
- 4.3. The level of derivatization was calculated as hydrazides per 100 kilo-daltons of dextran to facilitate the comparison between polymers of different average molecular weights [1].
 - 4.3.1. LAB MEDIA: Figure 4 C.

Conclusion

5. Conclusion Interview Statements

- 5.1. **Andrew Lees:** The protocol will need to be adapted for a specific polysaccharide. CDAP activation can be used to biotinylate polysaccharides. Using CDAP to functionalize polysaccharides allows the use of a wide variety of conjugation reagents.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1 for 'CDAP activation'*

- 5.2. **Andrew Lees:** Proteins can be directly linked to CDAP activated polysaccharides. This usually requires some optimization for each polysaccharide, but the conjugation yield can be over 75%. The use of CDAP has enabled low-cost conjugate vaccines, like the ones made by the Serum Institute of India.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.