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Polystyrene Surface-Absorbed Trehalose Diester as a Means for Performing Pulldown Assays

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Background

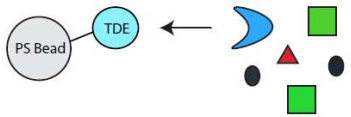
In 2016, over 10.4 million people contracted Tuberculosis (TB) infections for the first time, resulting in the deaths of 1.7 million people worldwide.[1] Despite the existence of a vaccine, TB remains the number one cause of death from a single infectious disease according to the 2017 report from the World Health Organization. As a result, research and development of a new TB vaccine is ongoing, as it is suspected the currently available formulation does not contain an adjuvant which produces the necessary TH-17 response. In the search for new drugs to combat this epidemic, knowledge of how a compound triggers an immune response is paramount. One method for identifying an activated immune protein complex is via immunoprecipitation, colloquially referred to as a "pulldown."

In this study, a pelletable, beaded support of polystyrene (PS) with surface-absorbed trehalose diester (TDE) has been developed for use as a tool for identification of C-type lectin receptors. TDE was used as a stand-in for trehalose dimycolate, a glycolipid found in the cell wall of *Mycobacterium tuberculosis*, which is recognized by C-type lectin. Confirmation of surface-absorption and characterization of the modified beads was performed via dynamic light scattering and high-performance liquid chromatography.

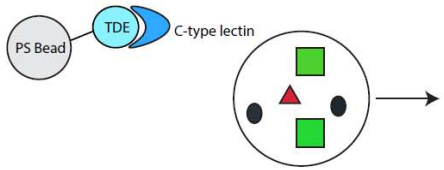
Pulldown Assay Process

Step 1: Attach TDE to PS-Bead

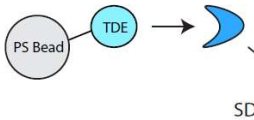
Step 2: Immobilize C-type lectin from cell lysate



Step 3: Spin down beads and wash away unbound species



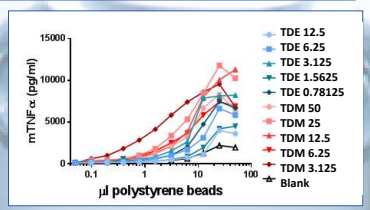
Step 4: Elute protein complex and indentify via SDS-PAGE analysis



Pulldown method developed from Kercher B, et al.(2016)[2]

PS-Bead Characterization

PS-beads were resuspended in a mannitol solution prior to lyophilization in order to provide maximum surface area for TDE absorption to occur evenly over the surface of the beads.



Dynamic Light Scattering Size Analysis

Sample	Z-Avg (nm)	PDI	Peak 1 Size (nm)	Peak 1 Vol. %	Peak 2 Size (nm)	Peak 2 Vol. %
Lot B4.1	114.8	0.416	144.6	1.2%	7.843	98.8%
Lot B4.2	119.5	0.32	135.4	33.3%	9.706	65.3%

RP-HPLC Concentration Analysis

Initial Loading Conc. µg/mL	Wash Assay Conc. µg/mL	Absorbed TDE Conc. µg/mL
200	9.3624	190.6376
200	13.2954	186.7046

Target characteristics

•Modified beads must be large enough to quickly pellet via centrifugation

•The surface of the beads must be uniformly coated by TDE

•The spreading density of TDE on the PS-beads must produce significant C-type lectin receptor binding

Results

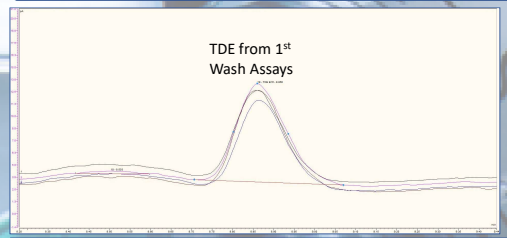
•5 micron beads pelleted quickly with only a small amount remaining suspended after 3 minutes of centrifugation or sitting undisturbed for less than one hour.

• A spreading density of 3.125 Å^2/molecule (15.41 µg/cm^2) produced the greatest receptor binding in RAW cells (labeled **TDE 3.125, top right**)

•Method for theoretically uniform surface-absorption of TDE developed (**picture top left**)

•TDE did not remain absorbed to bead surface throughout pulldown assay procedure

Reverse Phase HPLC

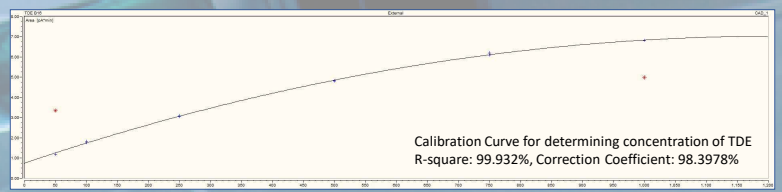


Mobile Phase Method

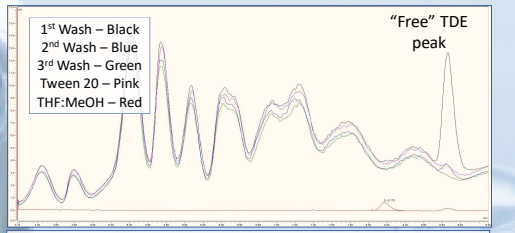
Time (min)	%A	%B	Flow (mL/min)
0	25	75	0.7
1	25	75	
4	0	100	
10	0	100	
11	25	75	
12	25	75	

A: 0.1% TFA
B: 0.1% TFA, in methanol

Actual concentrations of TDE absorbed to PS-beads were determined by indirect analysis of post-absorption washes via RP-HPLC. HPLC method information, including mobile phase composition and flow rate is given in the Table **(above right)**. This experiment provided consistent and clean data to determine concentration of free TDE remaining in samples of the modified PS-bead after three washes. Resulting concentrations were subtracted from the initial loading concentration to determine the final concentrations of TDE absorbed to the PS-beads **(above, under characterization header)**. Concentrations were calculated based upon an 8-point calibration curve with a R-square of 99.932% **(shown below)**.



Reverse Phase HPLC



This is a chromatogram of all 3 wash assays from Lot B4.1 with the 0.1% Tween 20 vehicle and 9:1 THF:MeOH **(above)**. TDE has completely disappeared by the third wash **(green)** although there is some carryover of TDE shown in the Tween and THF:MeOH samples, none was present in the final wash of the beads. All "free" TDE has successfully been removed from the suspension.

Conclusions & Future Directions

Conclusions

- TDE was successfully surface-absorbed to polystyrene beads at concentrations close to target
- Any TDE not absorbed was removed during post-absorption washes
- Resulting beads aggregate and pellet with short spin times or by sitting undisturbed for less than one hour.
- TDE did not remain absorbed to bead surface throughout pulldown assay procedure

Future Directions

- Develop further to prevent removal of TDE from PS-beads during pulldown: what causes the TDE to desorb from the PS-beads and how can this barrier be overcome?
- Calculate surface-absorbed TDE concentration directly by removing TDE from modified beads and analyzing via RP-HPLC

References

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Acknowledgements

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Background Image: [Photograph of vials and syringes] (2018). Retrieved from https://www.cancercenter.com/~/media/Images/Others/2018%20Online%20Publications/01-Jan/07-blog-Vaccines.jpg