AWARD NUMBER: PR182271 W81XWH-19-1-0041

TITLE: A milk protein-hitchhiking strategy for the oral delivery of amphiphilic vaccines

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REPORT DATE: MAY 2020

TYPE OF REPORT: Annual Progress Report Year 1

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE:	2. REPORT TYPE:	3. DATES COVERED
MAY 2020	ANNUAL	04/15/2019 to 04/14/2020

4. TITLE AND SUBTITLE

A milk protein-hitchhiking strategy for the oral delivery of amphiphilic vaccines

5a. CONTRACT NUMBER
PR182271

5b. GRANT NUMBER
W81XWH-19-1-0041

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Northeastern University
360 Huntington Avenue
Boston, MA, 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
NEU

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Oral vaccination is a very powerful and effective approach for vaccination, because it can not only induce a broad immunity through the blood and lymphatics, but also help patrol a very thin lining in the respiratory, digestive, and reproductive systems for potential virus invasion. The latter is particularly important as many pathogens invade the body via sexual or respiratory transmission. However, the stomach presents a major barrier to successful delivery of protein-based vaccines to the intestines, in which immune cells would otherwise receive alarm signals. The stomach is highly acidic and also contains proteins that recognize vaccines as food sources to chop up. To address this challenge, we draw inspiration from two seemingly irrelevant disciplines − dairy science and bioconjugation chemistry − to develop a "plug and play" type of approach for oral delivery of vaccines.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. Introduction

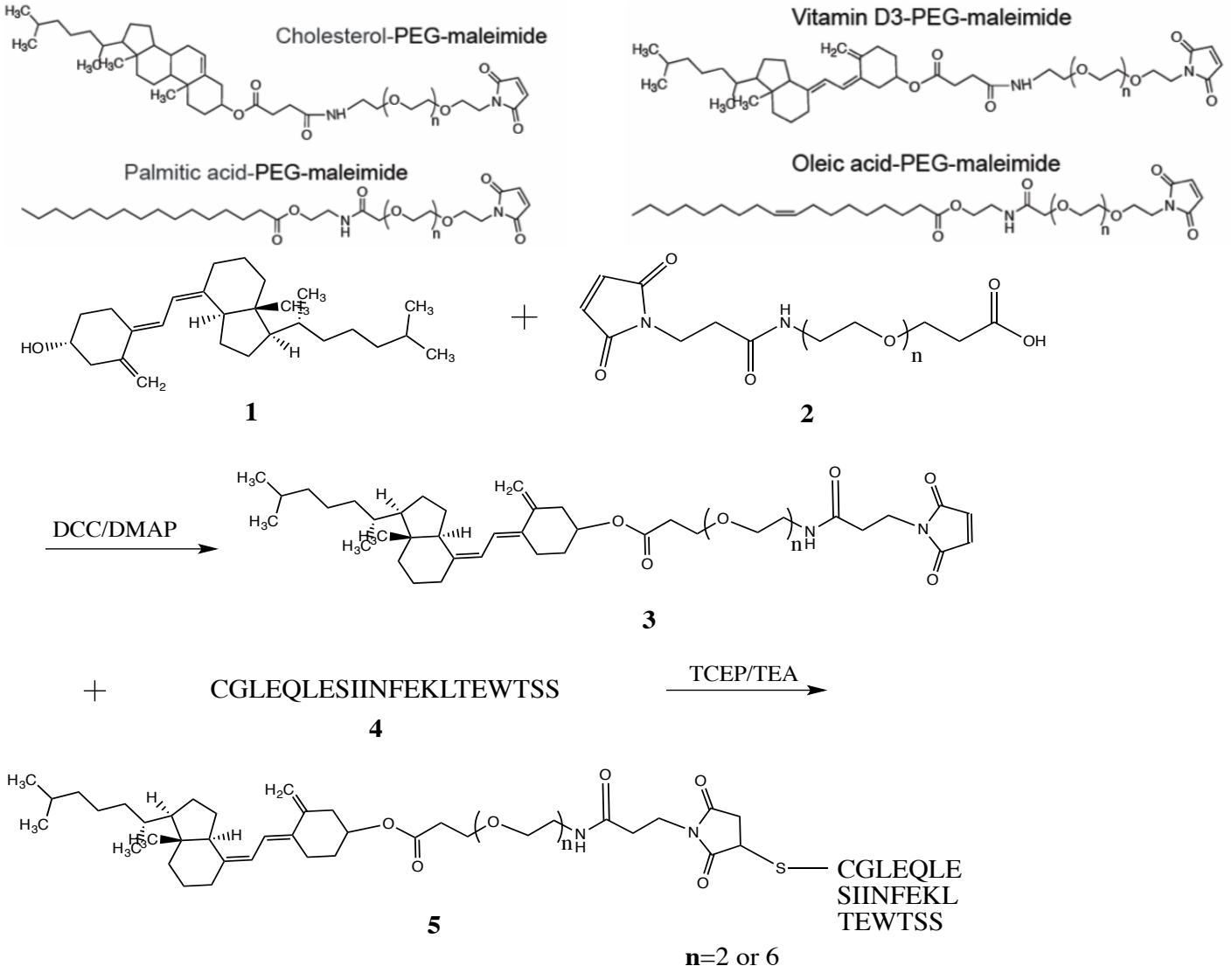
Service members and civilian populations are potentially exposed to many emerging infectious diseases that are life-threatening. These include, but are not limited to, Zika virus (ZIKV), dengue, HIV, norovirus, hepatitis, and Middle East Respiratory Syndrome. However, many of these diseases remain among the leading causes of illness and death in the battlefield, and account for substantial spending on the related consequences of infection even after soldiers come back from the service. Many infectious diseases, however, can be prevented through vaccination. Developing a convenient and cost-effective vaccine delivery technology that can be deployed to service members and civilians remains highly desirable. Oral vaccination is a very powerful and effective approach for vaccination, because it can not only induce a broad immunity through the blood and lymphatics, but also help patrol a very thin lining in the respiratory, digestive, and reproductive systems for potential virus invasion. The latter is particularly important as many pathogens invade the body via sexual or respiratory transmission. However, the stomach presents a major barrier to successful delivery of protein-based vaccines to the intestines, in which immune cells would otherwise receive alarm signals. The stomach is highly acidic and also contains proteins that recognize vaccines as food sources to chop up. To address this challenge, we draw inspiration from two seemingly irrelevant disciplines − dairy science and bioconjugation chemistry − to develop a “plug and play” type of approach for oral delivery of vaccines. Our strategy is to repurpose a milk protein, α-lactalbumin, as a vehicle, which is known to be resistant to degradation by stomach. Since this protein naturally associates with many bioactive lipids such as Vitamin D, we propose to explore a range of lipid molecules derived from a common supplement (e.g. Vitamin D) and vegetable oil (e.g. oleic acid). As a proof of principle study, we link lipid molecules to a short strand of amino acids (an antigenic peptide) that instructs the host immune system to mount potent humoral and cellular immune responses against the pathogen of interest. We envision that co-administering these two components with α-lactalbumin will help them from being cleared by the stomach, and therefore a stronger signal will be sent to immune cells lying underneath the intestines.

2. Keywords

Oral vaccine, subunit vaccine, α-lactalbumin, immunization

3. Accomplishments

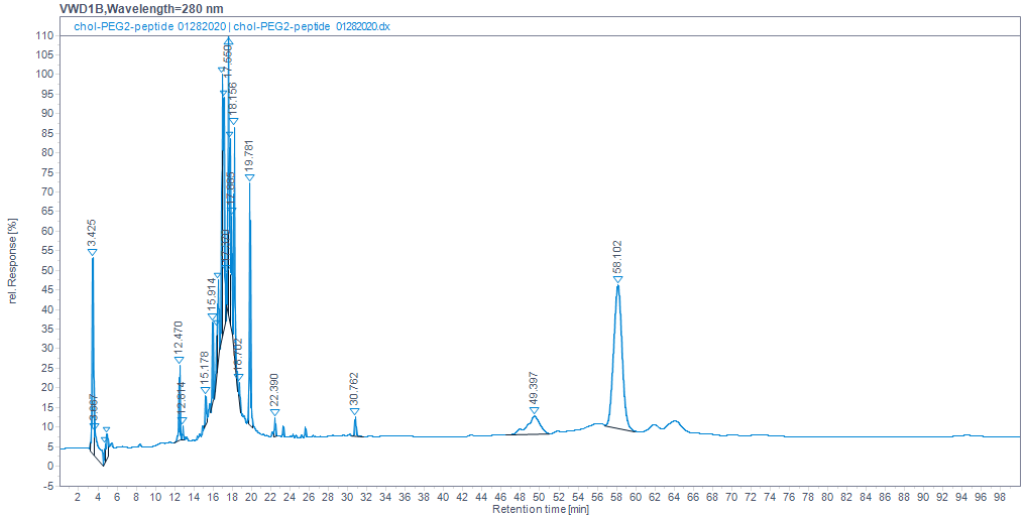
3.1 Synthesis of four different fatty acid-PEG-peptide conjugates bearing two different linker lengths



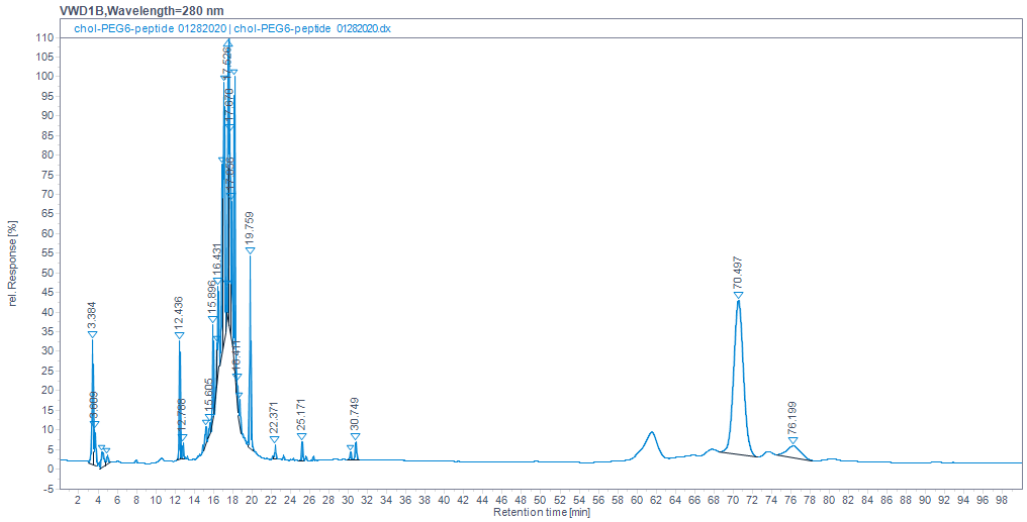
Scheme 1. Synthesis of VD3-PEG-peptide conjugations

To examine the affinity of a variety of natural and synthetic lipid molecules to α-lactalbumin, we selected four different lipids: vitamin D3, oleic acid, cholesterol and palmitic acid, based on their well characterized biological functions and different hydrophobicity. We propose to conjugate these lipophilic handles to a model epitope from chicken ovalbumin (SIINFEKL) with PEG2 and PEG6 as space linkers. Finally, eight different peptide conjugations were synthesized by a two-step chemical reaction (**Scheme 1**). For example, vitamin D3 (**1**) was firstly reacted with maleimide-NH-PEG6-CH2CH2COOH (**2**) to form the vitamin D3-PEG6-NH-maleimide (**3**) with DCC and DMAP as coupling agents. Then, vitamin D3-PEG2-peptide conjugation (**5**) were obtained by addition of sulfhydryl group of the peptide with vitamin D3-PEG6-NH-maleimide (**4**). Then the conjugation was purified and analyzed by high performance liquid chromatography (HPLC) and Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF). VD3-PEG6-peptide, Cholesterol-PEG2-peptide and Cholesterol-PEG6-peptide were also synthesized in the same way. On the other hand, the peptide derived from SIINFEKL with the sequence of GLEQLESIINFEKLTEWTSS, Oleic acid-PEG2-peptide, Oleic acid-PEG6-peptide, Pal-PEG2-peptide and Pal-PEG6-peptide were synthesized by solid phase peptide synthesis (SPPS) and purified by HPLC (**Figure 2**).

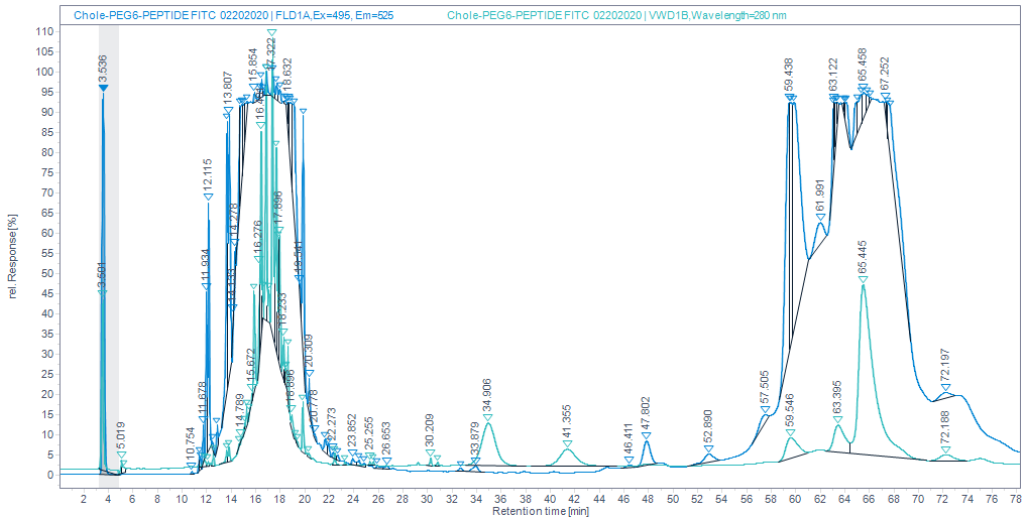
3.2 Purification and characterization of Cholesterol and Vitamin D-PEG2/6-Peptide



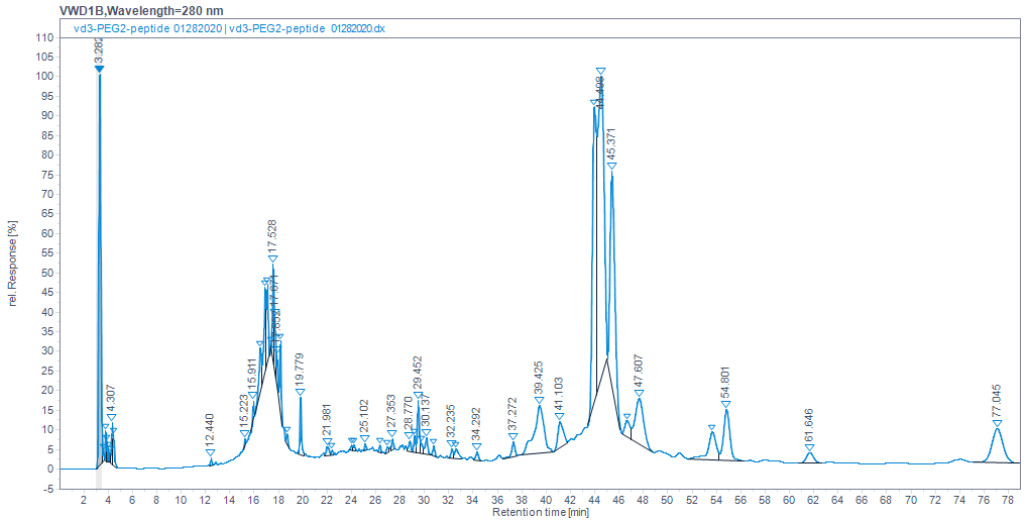
a. Cholesterol-PEG2-Peptide



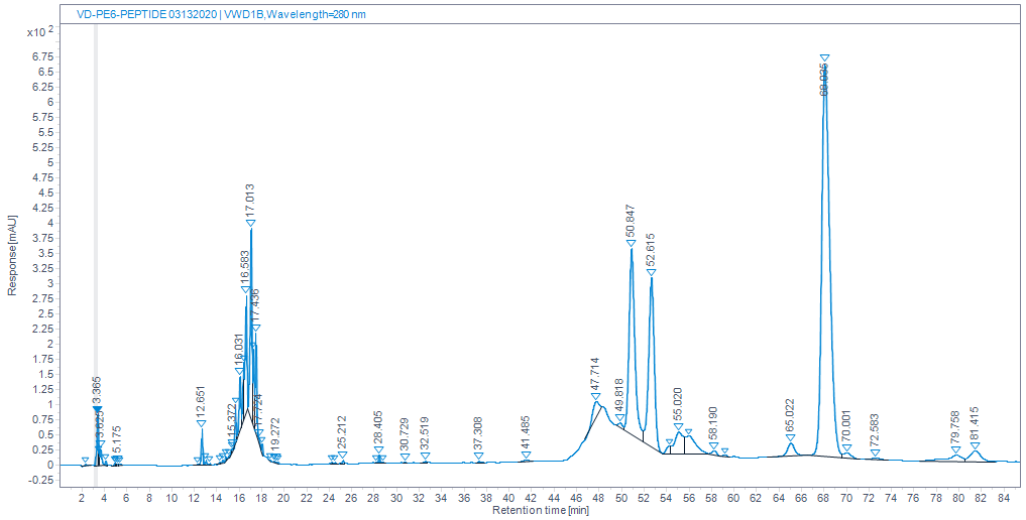
b. Cholesterol-PEG6-Peptide



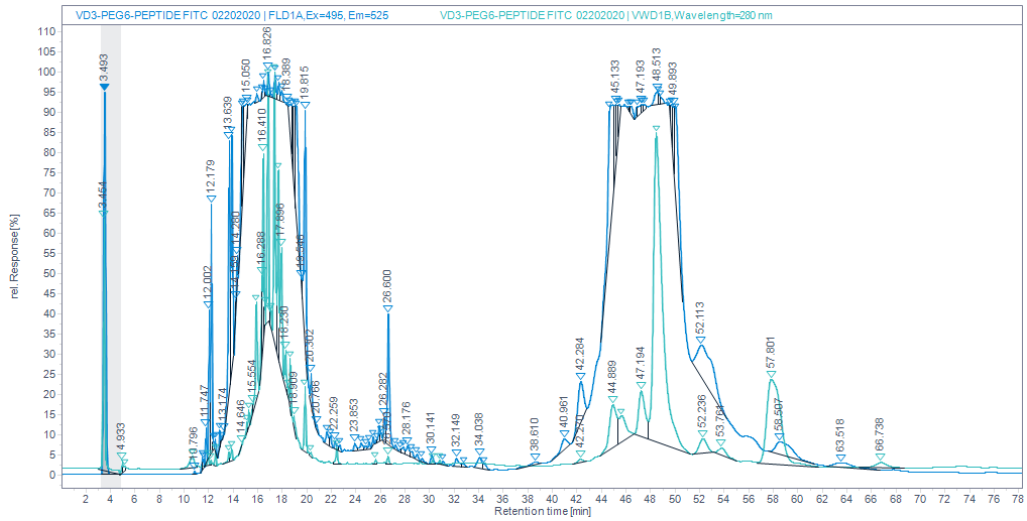
c. Cholesterol-PEG6-Peptide-FITC



d. Vitamin D3-PEG2-Peptide



e. Vitamin D3-PEG6-Peptide



f. Vitamin D3-PEG6-Peptide-FITC

Figure 2. Purification of fatty acid peptide conjugates by HPLC. a) The HPLC purification of Cholesterol-PEG2-Peptide, Cholesterol-PEG6-Peptide (b) and Cholesterol-PEG6-Peptide-FITC (c); d) The HPLC purification of Vitamin D3-PEG2-Peptide, Vitamin D3-PEG6-Peptide (e) and Vitamin D3-PEG6-Peptide-FITC (f).

3.3 *In vitro* induction of peptide-specific CD8 T cell proliferation by lipid-PEG-peptide conjugations

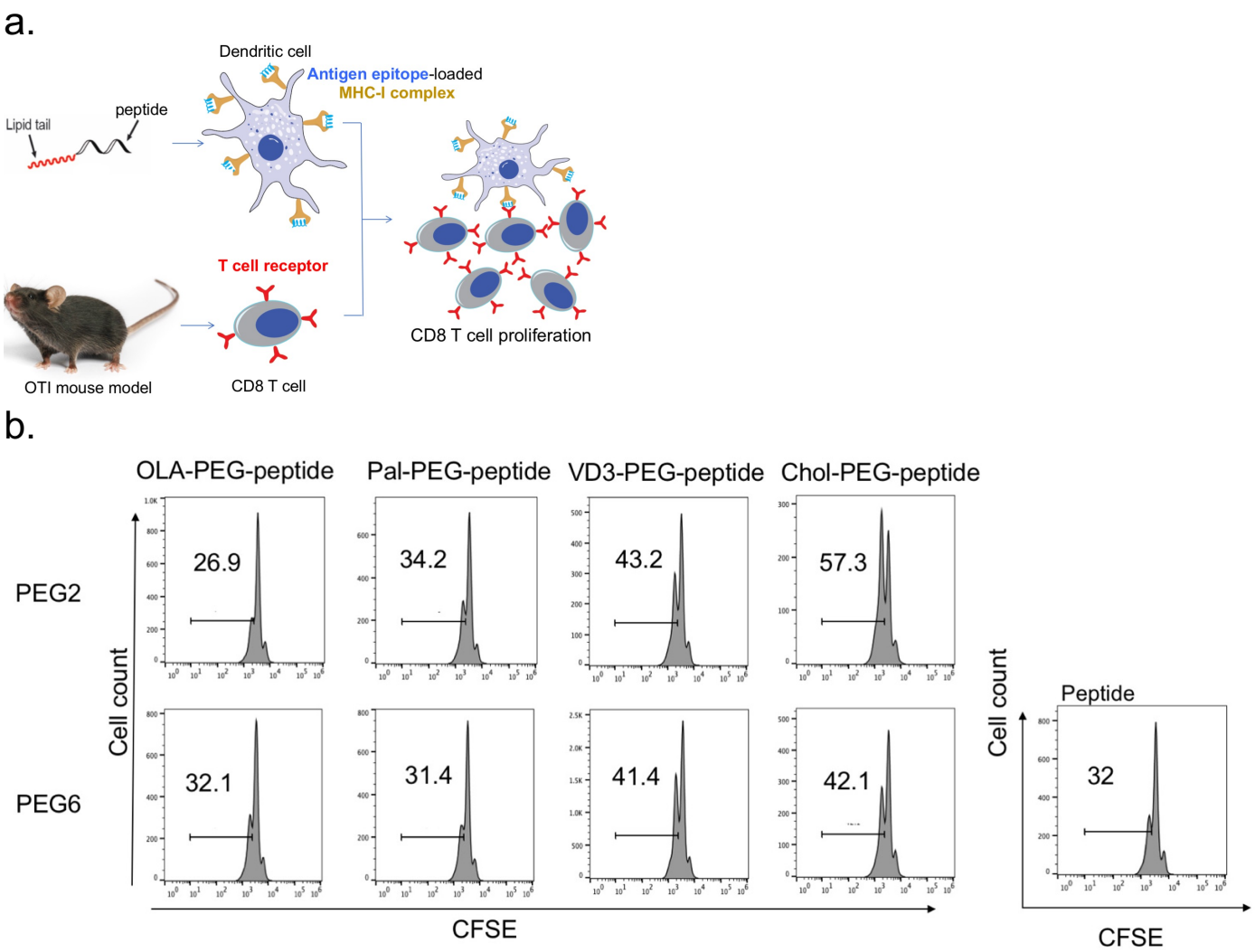


Figure 3 The ability of different lipid-PEG-peptide conjugations to activate the proliferation of OT-1 CD8 T cells. a) The schematic of proliferation of OT1-CD8 T cells induced by fatty acid peptide conjugates; b) The proliferation of OT-1 CD8 T cells after labeled by CFSE and incubated with Oleic acid (OLA)-PEG2-peptide, OLA-PEG6-peptide, Pal-PEG2-peptide, Pal-PEG6-peptide, Vitamin D3 (VD3)-PEG2-peptide, VD3-PEG6-peptide,Cholesterol (Chol) -PEG2-peptide, Cho-PEG6-peptide and peptide for 48 hr at 1μg/ml by flow cytometry.

In order to evaluate the potential of lipid-PEG-peptide conjugations to induce proliferation of SIINFEKL-specific CD8 T cells, an *in vitro* CD8 T cell proliferation was study based on OT-1 CD8 T cells, which carry a transgenic CD8 T cell receptor and can specifically recognize the peptide SIINFEKL. The OT-1 CD8 T cells were isolated from the lymph nodes of OT-1 mice and intracellularly labeled by carboxyfluorescein diacetate succinimidyl ester (CFSE) to track proliferating cells. It is worth noting that CFSE-labeled OT-1 CD8 T cells will clonally expand and the florescence of intracellular CFSE will be serially diluted after successfully stimulated by peptide antigen. **Figure 3** demonstrates the CFSE dilution of OT-1 CD8 T cells after incubation with eight different lipid-PEG-peptide conjugations and peptide for 48 h at 1 ug/ml by flow cytometry. CFSE signals of OT-1 CD8 T cells co-culture with SIINFEKL and synthesized peptide are serially decreased confirming that the addition of SIINFEKL with amino acids at both ends do not sacrifices its immunogenicity. Similarly, those of OT-1 CD8 T cells pulsed with lipid-PEG-peptide conjugations appear as a series of peaks with lower CFSE fluorescence intensities, suggesting that lipid-PEG-peptide conjugations successfully activate the CD8 T cells and the conjugation with lipid and PEG does not lower the peptide antigen immunogenicity. In contrast, the CFSE dilution of OT-1 CD8 T cells incubated with PBS is insignificant, indicating OT-1 CD8 T cells was not spontaneously stimulation in the absence of the peptide or peptide conjugates.

Moreover, to assess whether the lipid-PEG-peptide conjugation compromises its immunogenicity after complexation with lactalbumin, OT-1 CD8 T cell proliferation was quantified by incubation with lipid-PEG-peptide for 48h at 0.5 ug/ml in cell culture medium with/without lactalbumin (1:100 molar ration compared with peptide). Before co-incubation with OT-1 CD8 T cell, lipid-PEG-peptides were previously mixed with lactalbumin for 30 min. CD8 T cell exhibited comparable proliferation potentials when stimulated with lipid-PEG-peptides in the presence or absence of lactalbumin, indicating that the immunogenicity of lipid-PEG-peptides was maintained after binding with lactalbumin. In addition, CD8 T cells induced by lipid-PEG-peptides show enhanced proliferation ability when compared with that of peptide or SIINFEKL, suggesting that conjugation of peptide with lipid-PEG have enhanced immunogenicity than that of free peptide or SIINFEKL.

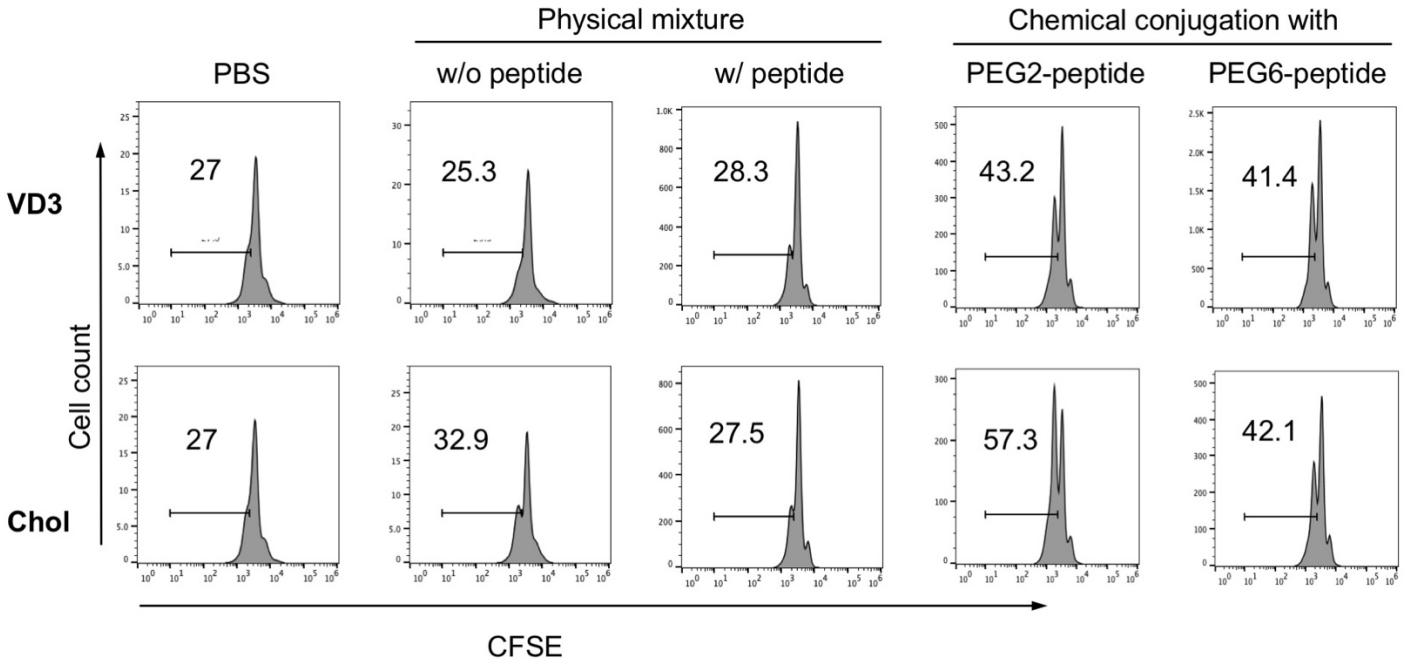


Figure 4. The ability of VD3 or Chol peptide conjugations and their physical mixture to proliferate the OT-1 CD8 T cells. OT-1 CD8 T cells were stimulated by vitamin D3 (VD3) or Cholesterol (Chol) peptide conjugations and their physical mixture at 1μg/ml of SIINFEKL peptide for 48 hr.

3.4 Uptake of peptide conjugates by mouse dendritic cells

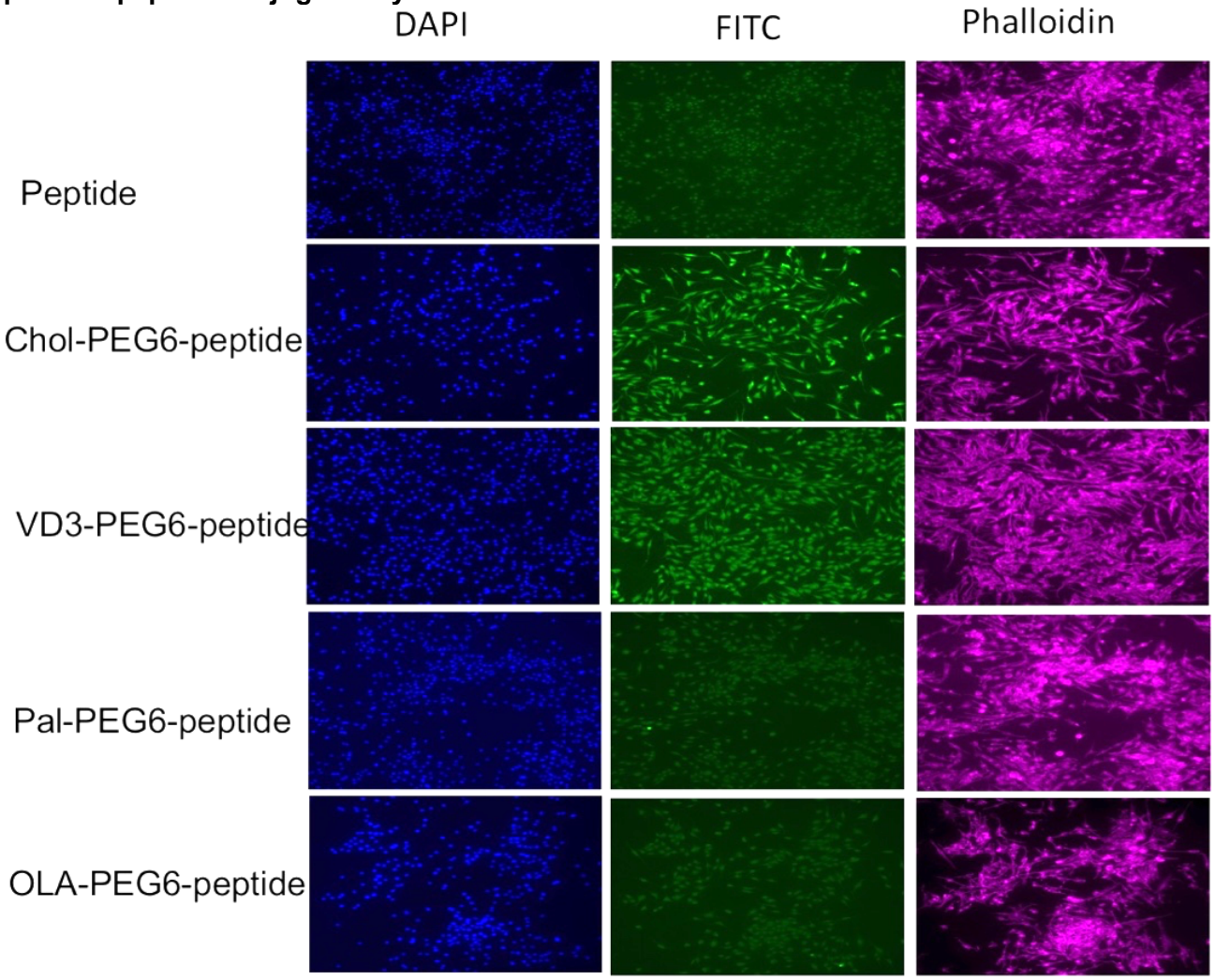


Figure 5. Fluorescent images of Lipid-PEG6-peptide conjugations or peptide uptake by DC 2.4 cells for 4h at 2.5 μg/ml. Chol/VD3/Pal/OLA-PEG6-peptide conjugations or peptide were labeled with fluorescein isothiocyanate (FITC, green). DC 2.4 cells skeleton were stained with a cytoskeleton-staining dye, phalloidin (magenta), and nuclei were stained by DAPI (blue). Abbreviations: cholesterol (Chol), VD3, vitamin D3; Pal, palmy

To evaluate if different conjugates can penetrate the cell membrane, their uptake was examined in the murine dendritic cell line D.C 2.4. **Figure 5** shows the fluorescent images of DC 2.4 cells after co-incubation with lipid-PEG-peptide conjugations or peptide only for 4 hr at 2.5 μg/ml. The cells with the VD3-PEG6-peptide and chol-PEG6-peptide conjugations showed higher fluorescent intensity than those with peptide, oleic acid-PEG6-peptide and pal-PEG6-peptide conjugates. The uptake of native peptide and conjugations were further confirmed by intracellular staining using flow cytometry (data not shown). Among of these conjugations, cholesterol-peptide demonstrated the highest uptake by DC 2.4 cells with vitamin D3 being the second highest, which is consistent with the fluorescence microscopy imaging results.

4. Impact

In summary, in year 1 of this funding, several peptide conjugates have been successfully synthesized and characterized by different chemical and biological assays including HPLC, mass spectrometry, fluorescence microscopy, flow cytometry, and immune cell assays. There different methods ensure the integrity and reproducibility of the compounds. We have completed the majority of Task 1 and Task 2 as outlined in our original Statement of Work. We are in the middle of preparing a provisional patent submission to that reports our in vitro synthesis and characterization, and we are also planning to submit a manuscript by the end of funding period.

5. Changes/Problems

In the first four months, we were trouble shooting the chemical synthesis as outlined in scheme 1. The synthesis turned out to be not as simple as we had originally thought. For example, it was difficult to purify the cholesterol-peptide conjugate from excess free cholesterol and we ended up using a preparation HPLC to separate them by paying a local facility to do a service for us. Additionally, when we synthesized vitamin D3-PEG6-NH-maleimide, we firstly used EDC and DMAP, but the conjugation efficiency of vitamin D3-PEG6-NH-maleimide was very low. After EDC were replaced by DCC, the conjugation efficiency of vitamin D3-PEG6-NH-maleimide were much improved.

We were planning to start a pilot animal study as outlined in Task 3 of SOW in April. Unfortunately, due to the pandemic shutdown, we were not able to access the laboratory until June. Now, we have reopened the campus and are planning to purchase C57BL/6 mice to run a pilot study by orally administering mice with different peptide conjugates to test their *in vivo* efficacy.

6. Products

During the pandemic lockdown, postdocs and students were actively participating in manuscript writing and revision. As a result, we have one review manuscript accepted and two research manuscripts published.

Journal articles:

Yang M, Zhu G, Korza G, Sun X, Setlow P, **Li J***. Engineering *Bacillus subtilis* as a Versatile and Stable Platform for Production of Nanobodies. Appl Environ Microbiol. 2020 Apr 1;86(8). doi: 10.1128/AEM.02938-19. Print 2020 Apr 1. PubMed Central PMCID: PMC7117931

He Y, Hong C, Yan EZ, Fletcher SJ, Zhu G, Yang M, Li Y, Sun X, Irvine DJ, **Li J***, Hammond PT. Self-Assembled cGAMP-STINGΔTM Signaling Complex as a Bioinspired Platform for cGAMP Delivery. 2020;6(24):eaba7589 (*** co-correspondence**)

Zhang X, Al-Dossary A, Hussain M, Setlow P, **Li J***. Applications of *Bacillus subtilis* Spores in Biotechnology and Advanced Materials, 2020, Accepted.

Presentation:

Zhang X, **Li J**. A milk protein-hitchhiking strategy for the oral delivery of amphiphilic vaccines. Work in Progress Seminar, May 16th, 2020. Department of Bioengineering, Northeastern University.

7. Participants & Other Collaborating Organizations

Name: Jiahe Li
Project Role: PI
Nearest Person-Month Worked: 1 month (summer) (Academic salary covered by NEU)

Name: Xiaopei Zhang
Project Role: Postdoc
Nearest Person-Month Worked: 6 months
Contribution to Project:

Name: Mengdi Yang
Project Role: Graduate Student
Nearest Person-Month Worked: 2 months (tuition is waived in the college of engineering at NEU)
Contribution to Project:

8. Special Reporting Requirements

9. **Appendices** Copies of papers published and in press are included in the Appendix