AWARD NUMBER: W81XWH-21-1-0135

TITLE: Resolution of Lymphedema with Induced Lymphangiogenesis Using Tissue Nanotransfection Technology

PRINCIPAL INVESTIGATOR: Aladdin H. Hassanein, MD

CONTRACTING ORGANIZATION: Trustees of Indiana University
980 Indiana Ave
Indianapolis, IN 46202-5130

REPORT DATE: March 2022

TYPE OF REPORT: Annual

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
March 2022	Annual	1MAR2021 - 28FEB2022

4. TITLE AND SUBTITLE

Resolution of Lymphedema with Induced Lymphangiogenesis Using Tissue Nanotransfection Technology

5a. CONTRACT NUMBER
W81XWH-21-1-0135

5b. GRANT NUMBER
PR200435

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Aladdin H. Hassanein

E-Mail: ahassane@iu.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Trustees of Indiana University
980 Indiana Ave
Indianapolis, IN 46202-5130

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)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Lymphedema is characterized by lymphatic vessel dysfunction causing accumulation of extracellular fluid in peripheral tissues resulting in limb enlargement. Lymphedema is on the spectrum of vascular anomaly diseases. Lymphedema can be primary (malformed lymphatic system) or secondary (acquired from traumatic injury, radiation, parasitic infection). Any inciting significant trauma to the lower extremities, groin, or axilla can lead to lymphedema. It is estimated that 20–40% of patients, including military veterans, who undergo treatment for solid malignancies such as breast cancer, melanoma, gynecological or urologic tumors, or sarcomas develop lymphedema. There is no cure for this progressive, life-long disease.

This study uses a novel, non-viral nanotechnology-based approach with tissue nanotransfection technology (TNT) to manage lymphedema by inducing lymphangiogenesis in a mouse tail model of lymphedema. TNT facilitates direct, transcutaneous gene delivery using a chip with nanochannels in a rapid (<100ms) focused electric field. The feasibility of TNT for in vivo gene delivery has been established and validated for other applications in animal models. This innovative method can potentially establish a clinically translatable new interventional paradigm to treat lymphedema by point-of-care focal upregulation of lymphangiogenic genes. Translational significance of this research project involves lymphangiogenesis using TNT being focal and not global. Furthermore, the technology is non-invasive, portable and can be administered in the field.

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			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

REPORT OUTLINE

1. **INTRODUCTION:**

Lymphedema is characterized by lymphatic vessel dysfunction causing accumulation of extracellular fluid in peripheral tissues resulting in limb enlargement. Lymphedema is on the spectrum of vascular anomaly diseases. Lymphedema can be primary (malformed lymphatic system) or secondary (acquired from traumatic injury, radiation, parasitic infection). Any inciting significant trauma to the lower extremities, groin, or axilla can lead to lymphedema. It is estimated that 20–40% of patients, including military veterans, who undergo treatment for solid malignancies such as breast cancer, melanoma, gynecological or urologic tumors, or sarcomas develop lymphedema. There is no cure for this progressive, life-long disease.

This study uses a novel, non-viral nanotechnology-based approach with tissue nanotransfection technology (TNT) to manage lymphedema by inducing lymphangiogenesis in a mouse tail model of lymphedema. TNT facilitates direct, transcutaneous gene delivery using a chip with nanochannels in a rapid (<100ms) focused electric field. The feasibility of TNT for *in vivo* gene delivery has been established and validated for other applications in animal models. This innovative method can potentially establish a clinically translatable new interventional paradigm to treat lymphedema by point-of-care focal upregulation of lymphangiogenic genes. Translational significance of this research project involves lymphangiogenesis using TNT being focal and not global. Furthermore, the technology is non-invasive, portable and can be administered in the field.

2. **KEYWORDS:** TNT, Lymphedema, Murine tail, Lymphatics

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

The following two aims were proposed.

Aim 1: Develop non-invasive, point-of-care topical tissue nanotransfection technology (TNT) based approach to manage lymphedema

Aim 2: Objectively validate efficacy of the novel approach utilizing live-animal, high resolution, two-photon intravital microscopy of lymphedematous murine tail.

The following table presents approved STATEMENT OF WORK with date of completion & status

Specific Aim 1: Develop non-invasive, point-of-care topical tissue nanotransfection technology (TNT) based approach to manage lymphedema	Timeline (Months)	Status
Task 1.1: *Focally Deliver Prox1, Slp-76, and, VEGF-C plasmids to murine tail lymphedema using TNT.*		
Obtain ACURO approval	1-3	Completed
Delivery of plasmids using TNT	3-12	Completed
Task 1.2: *Assessment of lymphangiogenesis in mouse tails treated with TNT-delivered Prox1, Slp-76, and, VEGF-C plasmids*		
Assess markers of lymphangiogenesis by using	12-24	Ongoing

immunohistochemistry		
Task 1.3: *Evaluation of lymphatic function in lymphedematous tails.*		
Subtask 1.3.1 Evaluation of lymphatic function by ICG near infrared laser angiography.	12-24	Ongoing
Subtask 1.3.2 Evaluation of phenotypic lymphedema with tail volume measurements.	12-24	Ongoing
Milestone(s) Achieved: Prox1 promotes lymphangiogenesis in murine tail model and it limits the development of postsurgical lymphedema		Completed
ACURO Approval	1-3	Completed
Successful application of TNT for gene delivery to murine tail model	3-12	Completed
Specific Aim 2: Objectively validate efficacy of the novel approach utilizing live-animal, high resolution, two-photon intravital microscopy of lymphedematous murine tail.		
Task 2.1: *Optimize two-photon intravital microscopy in murine tail model of lymphedema.*	3-12	Ongoing
Task 2.2: *Validate functional lymphedema resolution using novel approach of intra vital microscopy*	12-24	Ongoing
Milestone(s) Achieved: Successful validation of functional lymphatics in lymphedematous mice tail (post-TNT treatment)		
Optimization of intra vital microscopy on murine tail lymphedema	3-12	Ongoing
Validation of functional lymphangiogenesis using TNT	12-24	Ongoing

What was accomplished under these goals?

For this reporting period describe:

1. **Major Activities.** Towards major task 1, we have reported the successful focal delivery of plasmids Prox1, to the murine tail lymphedema using the Tissue NanoTransfection (TNT) technology. TNT based focal delivery of genetic cargo in murine tail lymphedema was validated by using immunohistochemistry and qRT-PCR. TNT-delivered Prox1 significantly prevents postsurgical lymphedema determined by mice tail volume measurements. Also, the TNT_{Prox1} animals had significantly faster lymphatic drainage as measured by indocyanine green clearance and increased lymphatic vessel density as measured by Prox1 immunohistochemistry ($p<0.05$).

2. **Specific Objectives**

Major Task 1: Develop non-invasive, point-of-care topical tissue nanotransfection technology (TNT) based approach to manage lymphedema.

Subtask 1.1: Focally Deliver Prox1, Slp-76, and VEGF-C plasmids to murine tail lymphedema using TNT.

Subtask 1.2: Assessment of lymphangiogenesis in mouse tails treated with TNT-delivered Prox1, Slp-76, and, VEGF-C plasmids.

Subtask 1.3: Evaluation of lymphatic function in lymphedematous tails.

Major Task 2: Objectively validate efficacy of the novel approach utilizing live-animal, high resolution, two-photon intravital microscopy of lymphedematous murine tail.

Subtask 2.1: Optimize two-photon intravital microscopy in murine tail model of lymphedema.

Subtask 2.2: Validate functional lymphedema resolution using novel approach of intra vital microscopy.

3. Significant Results

Major Task 1

Develop non-invasive, point-of-care topical tissue nanotransfection technology (TNT) based approach to manage lymphedema

[Proprietary Data I] Subtask 1.1 Focally Deliver Prox1, Slp-76, and VEGF-C plasmids to murine tail lymphedema using TNT.

Tissue nanotransfection (TNT) devices were fabricated from thinned (~200 µM) double-side polished (100) silicon wafers using standard cleanroom fabrication technologies (**Fig. 1**). Briefly, a ~1.5 µm thick layer of AZ5214E was spin coated on wafer surface. Nanopores were subsequently patterned on the photoresist *via* projection lithography. Such pores were then used as etch masks to drill ~10 µm deep nanochannels on the silicon surface by deep reactive ion etching (DRIE) using a combination of SF6/C4F8 gases. The murine tail model of lymphedema was utilized. A 3 mm full thickness skin excision and lymphatic vessel disruption was performed 20 mm from the base of the mice. TNT was applied to the murine tail (day 0) directly at the surgical site with genetic cargo loaded into the TNT reservoir: Group I (control) was given pCMV6 (expression vector backbone alone) (n=6); Group II had pCMV6-Prox1 (n=6). TNT was applied with square wave pulse electric stimulation (10x10ms pulses, 250 V, 10 mA). The efficiency of gene delivery was assessed through qRT-PCR using primers with SYBR Green fluorescence quantification and immunostaining with anti-Prox1 antibody. TNT_{Prox1} group exhibited four-fold increased expression of Prox1 using qRT-PCR compared to TNT_{Sham} group at the site of TNT treatment (P=0.002). Increased expression of Prox1 was also observed with immunohistochemistry post-TNT (day 3) application. Intensity quantification of immunohistochemistry revealed greater expression of Prox1 in TNT_{Prox1} when compared to TNT_{Sham} (P=0.002).

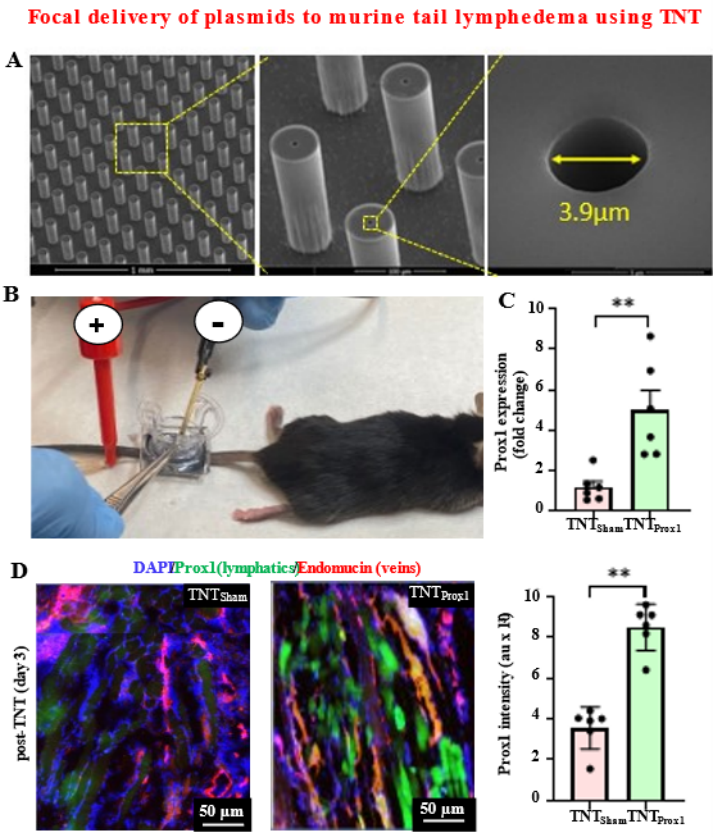

Figure 1. A. Scanning electron microscopy of $TNT_{2.0}$ showing the nano-needles and pore diameter 3.9µm. **B.** Topical delivery of pCMV6-Prox1 using $TNT_{2.0}$ in mice tail model on the day of surgery (d0). **C-D.** Validation of pCMV6-Prox1 delivery and expression through (C) q-RT PCR (D) immunostaining with anti-Prox1 antibody.

[Proprietary Data II] Subtask 1.2. Assessment of lymphangiogenesis in mouse tails treated with TNT-delivered Prox1, Slp-76, and VEGF-C plasmids Immunohistochemistry

was performed to assess lymphangiogenesis marker using antibody to Prox1 (PA5-26170, Invitrogen, Thermo Fischer). A comparison was performed between the TNT_{Prox1} and TNT_{Sham} group in C57BL/6 mice at post-TNT (day 28). Increased expression was observed in TNT_{Prox1} group when compared to TNT_{Sham} group (**Fig. 2**). Intensity quantification of immunohistochemistry revealed greater expression of Prox1 in TNT_{Prox1} when compared to TNT_{Sham} (P=0.002). This increased expression of Prox1 clearly depicts that the increased lymphatic vessel density in the TNT_{Prox1} group.

Assessment of lymphangiogenesis in mouse tails treated with TNT-delivered Prox1

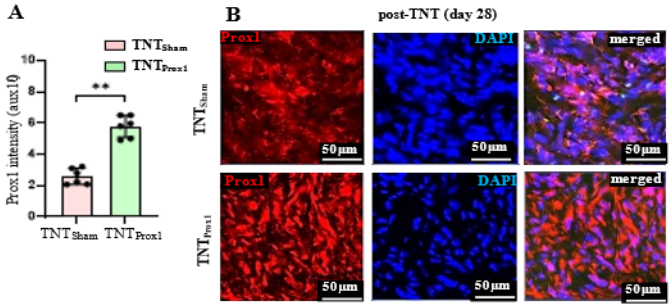

Figure 2. A-B. Increased expression of lymphatic marker (Prox1) as quantified using immunochemistry with anti-Prox1 (red) in C57BL/6 mice (Post-TNT-day 28). Data presented as mean ± SD.

[Proprietary Data III] Subtask 1.3. Evaluation of lymphatic function in lymphedematous tails.

ICG lymphangiography is a well-established method to visualize lymphatic vessels and display lymphatic clearance in real time. In normal (unoperated) murine tail, an intradermal injection of ICG results in rapid visualization of lymphatic vessels and rapid drainage of the fluorescent dye. Whereas, ICG injection (same volume) in lymphedema induced murine tail (post-op) results in diffuse dermal backflow that persists for >48 hours. Interesingly, a consistent and characteristic speckling pattern is noted in the ICG drainage of lymphedematous mice tail over time.

We found the observed difference in lymphedema with TNT_{Prox1} treatment and it may be due to the improved lymphatic drainage. When we compared the lymphedema induced mice tail of TNT_{Sham} vs TNT_{Prox1}, we found that TNT_{Sham} animals had persistence of fluorescent signal for up to 96 hours. ICG clearance was significantly faster in the TNT_{Prox1} treated animals at 48, 72, and 96 hours. The half-life of ICG clearance was significantly less with TNT_{Prox1} ($t_{1/2}$ = 60 vs. 102 hours).

Prox1 delivery resulted in a statistically significant difference in relative mice tail volume compared with control animals at time point of 14 and 28 days. TNT_{Prox1} animals has less early postsurgical edema and significantly less tail lymphedema compared with TNT_{Sham} animals.

Other Achievements.

Nothing to report.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

We plan to present research findings in peer-reviewed scientific and medical journals to ensure that the results from the project are disseminated as widely as possible. A manuscript will be submitted with the findings once all data is finalized. We have submitted the abstracts to the related scientific meetings such as MHSRS/PSRC/ACS.

What do you plan to do during the next reporting period to accomplish the goals?

We will continue working on characterisation of functional induced lymphangiogenesis in the TNT_{Prox1}-treated lymphedematous mice tail by using novel intravital two-photon microscopy (IVM).

4. IMPACT:

What was the impact on the development of the principal discipline (s) of the project?

Short term impact – Development of focal lymphangiogenesis using TNT will be a novel therepeutic approach towards lymphedema management. It is non-invasive, portable and can be administered topically.

Long term impact – The project aims to resolve lymphedema associated with filariasis, traumatic injuries or cancer treatment by inducing functional lymphangiogenesis. The treatment will be facilitated *via* delivery of lymphangiogenic genes through tissue nanotransfection technology (TNT). Because of the ease of point-of-care, focal, delivery through the skin at the affected site, we anticipate that it will be used for clinical applications. It will applicable for military with lymphedema from traumatic injury, parasitic infection that causes lymphedema, and cancer treatment as well as the 5 million civilian Americans with lymphedema. Therefore, the ability to treat such individuals to facilitate full functional recovery and improved quality of life is a key goal visioned through this proposal.

What was the impact on other disciplines?

Research outcomes will be leveraged towards the establishment of a sustainable research program based on the novel concept of nanotechnology-based induction of lymphangiogenesis in lymphdema models. If successful, the non-invasive, point-of-care topical tissue nanotransfection technology (TNT) based approach will be used to manage lymphedema. Because of the simplicity and safety of the technology, we anticipate that it will be used for clinical applications other than lymphedema in which topical delivery of genetic cargo may be benficial.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Completion of the proposed study will greatly benefit care of military lymphedema patients with chronic conditions and could significantly reduce cost and burden to the DoD and VA healthcare systems by providing justification towards the use of direct, *in vivo* TNT technology to manage lymphedema. Furthermore, clinicians working in Military Treatment Facilities (MTFs), Veterans Health Administration (VHA), as well as those in academic and general medical facilities, will gain needed information regarding next generation therapeutics for lymphedema management.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to Report
Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report
Changes that had a significant impact on expenditures
Nothing to Report
Signficant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects
NA
Significant changes in use or care of vertebrate animals
No changes
Significant changes in use of biohazards and/or select agents
NA

6. PRODUCTS:

PUBLICATIONS:

1. Cook JA, Sinha M, Lester M, Fisher CS, Sen CK and Hassanein AH. Immediate Lymphatic Reconstruction to Prevent Breast Cancer-Related Lymphedema: A Systematic Review. *Adv Wound Care (New Rochelle)*. 2022.
2. Hassanein AH, Sinha M, Neumann CR, Mohan G, Khan I and Sen CK. A Murine Tail Lymphedema Model. *J Vis Exp*. 2021.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

- Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanges from a previous submission, provide the name only and indicate “no change.”

Name:	Dr. Aladdin Hassanein
Project Role	PI
Person month per year	5%
Name:	Dr. Mithun Sinha
Project Role	Co-I
Person month per year	2%
Name:	Dr. Ganesh Mohan
Project Role	Postdoctoral Fellow
Person month per year	20%

- Has there been a change in the active other support of the PD/Pi9s) or senior/key personnel since the last reporting period?
  Nothing to Report

- What other organisations were involved as partners?
  Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS
Nothing to Report

9. APPENDICES: NA