AWARD NUMBER: W81XWH-19-1-0120

TITLE: Targeted Nanobubble Technology to Control Diabetic Macrophage Function

PRINCIPAL INVESTIGATOR: Dr. Sashwati Roy

CONTRACTING ORGANIZATION: Trustees of Indiana University

REPORT DATE: April 2021

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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# REPORT DOCUMENTATION PAGE

*Form Approved*
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| 1. REPORT DATE | 2. REPORT TYPE | 4. DATES COVERED |
|---|---|---|
| April 2021 | 3. Annual Report | 04/01/2020-04/30/2021 |

**5. TITLE AND SUBTITLE**

Targeted Nanobubble Technology to Control Diabetic Macrophage Function

**5a. CONTRACT NUMBER**
W81XWH-19-1-0120

**5b. GRANT NUMBER**
**PR182424**

**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)**

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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)**
CDMRP

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

**12. DISTRIBUTION / AVAILABILITY STATEMENT**

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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

Purpose & Scope. The use of micro- and nanobubble systems combined with ultrasound (US) for drug and gene delivery has gained attention because the US can be used to trigger and enhance delivery via sonoporation. The objective of the proposed research is to develop a novel approach of miR-21 mimic cargo delivery using targeted gas nonobubbles. The delivery is anticipated to improve plasticity of injury-site macrophages in diabetic ulcers thus, facilitating resolution of inflammation and promote wound healing. Results & Significance. I) Successfully formulated macrophage targeted cationic nanobubbles (mNB) which are sufficiently stable to allow for adequate therapy time. II) optimized the delivery of mNB in excisional wounds that was trackable under US imaging using Vevo2100. III) The delivery resulted in improved wound closure in a delayed wound healing mice model of miR-21lysM cre. These data re now finalized. This year, the focus was to finalize the diabetic mice wound NB delivery and effect on healing outcomes as well as the resolution of inflammation. The specificity of macrophage targeted NB taken up by human macrophages as compared to keratinocytes. Furthermore, we determined if busting NB with ultrasound waves improves the delivery of miR-21 in human macrophages.

**15. SUBJECT TERMS**

Nanobubbles, miRNA delivery, diabetic wounds, inflammation, macrophage plasticity

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
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| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRDC |
| Unclassified | Unclassified | Unclassified | Unclassified | 9 | 19b. TELEPHONE NUMBER *(include area code)* |

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# REPORT OUTLINE

1. **INTRODUCTION:**

The use of micro- and nanobubble systems combined with ultrasound (US) for drug and gene delivery has gained attention because the US can be used to trigger and enhance delivery via sonoporation. Macrophages at the site of injury are known to be plastic. This plasticity of macrophages can be induced through delivery of reprogramming factors to macrophages. Loss in macrophage plasticity resulting in stalled inflammation is one of the primary causes of non-resolving ulcers in diabetics. Nanobubbles provide a promising non-viral strategy for US mediated gene delivery. This technique presents a variety of advantages, including local applicability and proven safety. The objective of the proposed research is to develop a novel approach of miR-21 mimic cargo delivery using targeted gas nonobubbles. The delivery is anticipated to improve plasticity of injury-site macrophages in diabetic ulcers thus, facilitating resolution of inflammation and promote wound healing.

2. **KEYWORDS:** Nanobubbles, miRNA delivery, diabetic wounds, inflammation, macrophage plasticity

3. **ACCOMPLISHMENTS:**

**What were the major goals of the project?**

The following specific aims were proposed:

Specific Aim 1- Develop and ultrasound-based tracking and delivery of macrophage targeted gas nanobubbles carrying miR-21 mimic cargo ($NB^{m\phi}$-miR-21).

Specific Aim 2-Test whether $NB^{m\phi}$-miR-21 rescues injury-site macrophage plasticity in diabetic wounds and improves the resolution of inflammation and healing.

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

| **Specific Aim 1(specified in proposal):** | **Timeline** Months | **Actual date of completion** | **Status** |
|---|---|---|---|
| **Major Task 1 develop macrophage targeting gas nanobubbles carrying miR-21 mimic cargo.** | | | |
| Subtask 1.1: *MicroRNA-NB Conjugation Assay* | 1-3 | Sept 2019 | Completed |
| Subtask1.2: *Characterization of NB properties: size distribution, concentration, buoyancy, and charge* | 1-6 | Dec 2019 | Completed |
| Subtask1.3: *Ultrasound-triggered gene delivery* | 6-12 | Mar 2020 | Completed |
| **Milestone(s) Achieved** | | | |
| 1. ILACUC approval<br>2. Macrophage targeted gas nanobubbles carrying miR-21 ready for in vivo applications | 1-3<br>12 | June 2019<br>Feb 2020 | Complete<br>Complete |
| **Specific Aim 2** | | | |
| **Major Task 2: Testing the efficacy of macrophage targeted nanobubbles carrying miR-21 mimic cargo modifying injury-site macrophage plasticity and improving resolution of inflammation and healing in diabetic wounds.** | | | |
| Subtask 2.1. *Delivery and tracking of miR-21 to diabetic wound-site macrophages in an excisional wound model.* | 6-12 | Ongoing | 90% complete |
| Subtask 2.2 *Confirm if delivery miR-21 to wound-site macrophages improves macrophage plasticity* | 6-18 | ongoing | 60% |

| | | | |
|---|---|---|---|
| *and facilitates resolution of wound inflammation healing.* | | | complete |
| **Milestone(s) Achieved:** | | | |
| The delivery of macrophage targeted nanobubbles carrying miR-21 that modify macrophage plasticity and improves resolution of inflammation and healing in diabetic wounds is achieved. | 18 | Ongoing | 75% complete |

## What was accomplished under these goals?

*For this reporting period describe:*

**1) major activities.** Towards major task1, we reported development of macrophage targeting gas nanobubbles (NB) carrying miR-21 mimic cargo in year-1. This included preparation, characterization and optimization platform cationic NBs which are needed to carry the miR-21 cargo. This reporting period, We focused on characterizing specificity of the NB in targeting macrophages using FAM DNA as a fluorescent mimic of genetic material. Furthermore, we performed task 1.3 - Ultrasound-triggered gene delivery & tracking.

Major Task 2, our primary focus was to complete the delivery of miR-21 to diabetic wound-site macrophages in an excisional wound model (subtask 2.1) and conducted final experiments to confirm if delivery miR-21 to wound-site macrophages improves macrophage plasticity and facilitates resolution of wound inflammation healing (sub task 2.2).

**2) specific objectives**

a. Major Task 1: develop macrophage targeting gas nanobubbles carrying miR-21 mimic cargo.
- Subtask 1.1: MicroRNA-NB Conjugation Assay
- Subtask1.2: Characterization of NB properties: size distribution, concentration, buoyancy, and charge
- Subtask1.3: Ultrasound-triggered gene delivery

b. Major Task 2: Testing the efficacy of macrophage targeted nanobubbles carrying miR-21 mimic cargo modifying injury-site macrophage plasticity and improving resolution of inflammation and healing in diabetic wounds.
- Subtask 2.1. Delivery and tracking of miR-21 to diabetic wound-site macrophages in an excisional wound model.
- Subtask 2.2 Confirm if delivery miR-21 to wound-site macrophages improves macrophage plasticity and facilitates resolution of wound inflammation healing.

**3) significant results**

**Major Task 1**

**develop macrophage targeting gas nanobubbles carrying miR-21 mimic cargo.**

**[Proprietary Data I]** *Subtask1.1 MicroRNA-NB Conjugation Assay*. This subtask was partially completed in year -1 and reported, we continued to test successful conjugation of miRNA with NB using human blood monocyte derived macrophage (MDM) system. Delivery of macrophage targeted miR-21 carrying NB, for 6-12h. The uptake of NB was evident already 6h post-delivery, however, uptake was ~4 fold higher as compared to uptake at 6h (**Fig 1**). To determine the specificity of the uptake of macrophage targeted NB by human MDM versus human keratinocytes, the cells were treated with macrophage

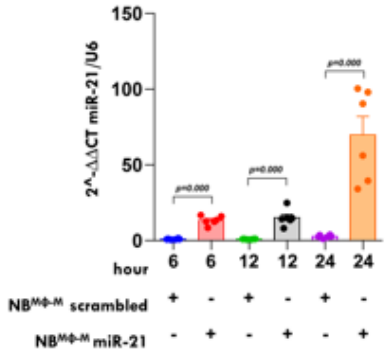


**Figure 1. Optimization of time dependent-uptake of $NB^{m\phi\text{-}M}$ -miR21 by human MDM.** Human blood monocyte derived macrophages (MDM) were treated with either macrophage targeted nanobubbles carrying miR-21 cargo ($NB^{m\phi\text{-}M}$-miR21) or $NB^{m\phi\text{-}M}$ containing control (scrambled) cargo ($NB^{m\phi\text{-}M\ scram}$) for 6-24h. The cells were harvested, and miR-21 abundance was determined using RTPCR. Data is mean±SD (n=6).

targeted nanobubbles carrying FAM (green) cargo ($NB^{m\phi\text{-}M}$-FAM) for 24h. The cells were countered stained with CD11b- PE (macrophages, red) or K14- alexa 405 (keratinocytes, blue) immunofluoscence microscopy was performed. The data clearly indicates a specific uptake of green NB by MDM (**Fig 2**).

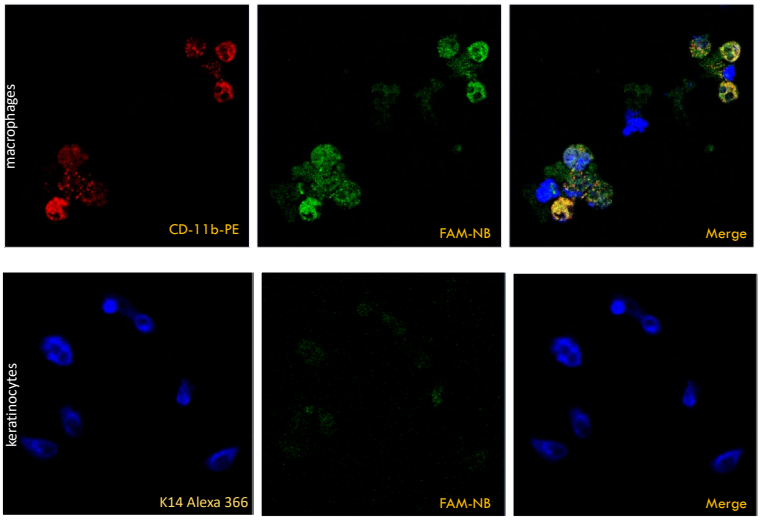


**Figure 2. Preferantial uptake of FAM labeled (green) $NB^{m\phi\text{-}M}$ by human MDM.** Human blood monocyte derived macrophages (MDM) or keratinocytes were treated with macrophage targeted nanobubbles carrying FAM (green) cargo ($NB^{m\phi\text{-}M}$-FAM) for 24h. The cells were countered stained with CD11b- PE (macrophages, red) or K14- alexa 405 (keratinocytes, blue) immunofluoscence microscopy was performed.

**[Proprietary Data II]** *Subtask1.2. Characterization of NB properties*: size distribution, concentration, buoyancy, and charge. This has been completed and reported in Year 1 Annual report.

**[Proprietary Data III]** *Subtask1.3: Ultrasound-triggered gene delivery:* One advantage of the NBs is their ability to be easily destroyed noninvasively **with ultrasound**. This destruction can lead to permeabilization of the endothelium and cell membranes, which in turn, facilitates the delivery of genetic material to the target cells. According to prior studies, an effective sonoporation protocol includes 60 s ultrasound stimulation (at 50-250 kPa, 1 MHz, 40% DC, 10 kHz pulse repetition frequency). To determine efficacy of ultrasound (US)-triggered miRNA-21 delivery, we utilized a moue macrophage culture system, where macrophages were cultured for 24h in presence of macrophage targeted NB carrying miR-21 or control scrambled miRNA. Following 24h, the cells were subjected to US mediated sonoporation (three bursts) using a Mettler Electronics Sonicator 740x ultrasound unit (Fig 3). miR-21 levels in cells was determined using RTPCR. The data suggest no added benefit of ultrasonication on the delivery of miR-21 to mouse macrophages.

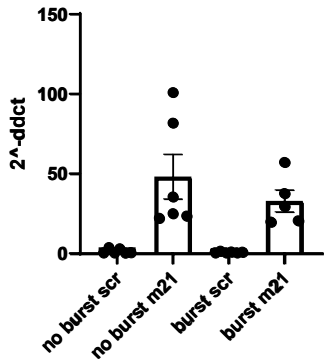


**Figure 3**. Macrophages were cultured for 24h in presence of macrophage targeted NB carrying miR-21 or control scrambled (scr, control) miRNA. Following 24h, the cells were subjected to US mediated sonoporation (three bursts) using a Mettler Electronics Sonicator 740x ultrasound unit. miR-21 levels in cells was determined using RTPCR

**Major Task 2: Testing the efficacy of macrophage targeted nanobubbles carrying miR-21 mimic cargo modifying injury-site macrophage plasticity and improving resolution of inflammation and healing in diabetic wounds**

**[Proprietary Data IV]** Effect of Macrophage targeted miR-21 carrying NB delivery on the wound healing in diabetic (db/db) mice was determined. The diabetic mice wounds were treated intradermally either with macrophage targeted NB carrying miR-21 ($NB^{m\phi}$-miR21) or macrophage targeted NB carrying scrambled (control) miRNA ($NB^{m\phi}$-scr) to any improvement in wound closure (**Figure 4**). Preliminary analysis indicated a beneficial effect of mNB-miR-21 delivery in d/db wound closure.

## 4) 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?

*Nothing to report.*

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 plan to submit abstracts for related scientific meetings such as MHSRS/WHS.

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

*We plan to conclude all studies by July and finalize all data analysis within the "no cost extension" period for a publication in a reputed scientific journal.*

4. **IMPACT:**

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

The proposed technology will help in resolving inflammation and improve diabetics. Such effect will be mediated via delivery of genes through targeted gas nanobubbles. Because of the simplicity of the technology and safety of the gas nanobubbles, we anticipate that it will be used for clinical applications especially topical application for wounds.

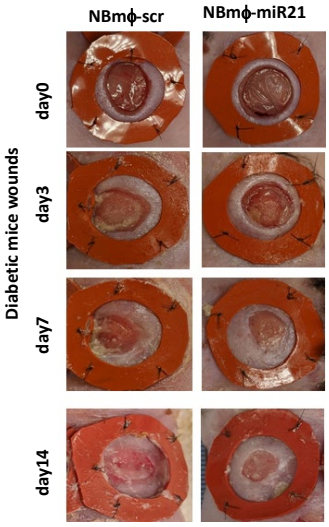


**Figure 4.** The **diabetic (db/db) mice wounds** were treated intradermally either with macrophage targeted NB carrying miR-21 (NBmϕ-miR21) or macrophage targeted NB carrying scrambled (control) miRNA (NBmϕ-scr). Wound images on day0-14 post-wounding have been presented.

### What was the impact on other disciplines?

Completion of the proposed study will greatly benefit care of military patients and could significantly reduce cost and burden to the DoD and VA healthcare systems by providing justification towards the use of a direct, in vivo reprogramming technology for diabetic complications like non-healing wounds. 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 treating uncontrolled inflammation.

### What was the impact on technology transfer?

Nothing to Report

Once we finalize the preclinical data with diabetic ulcers that appears promising, we plan to file an IP disclosure.

### What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:**

**Actual or anticipated problems or delays and actions or plans to resolve them**

There were delays that we faced because of COVID-19 related closure of Research facilities and quarantine of personnel. The diabetic mice delivery required for Aim 2 was affected. Because of the delay, we are requested a no cost extension.

**Changes that had a significant impact on expenditures**

COVID-19 related closure is anticipated to affect the financial health (personnel expenses incurred in the closure period) of the project.

### Significant 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:

Nothing to Report

## 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 unchanged from a previous submission, provide the name only and indicate "no change."*

| | |
|---|---|
| Name: | Dr. Sashwati Roy |
| Project Role: | PI (IU) |
| person month per year | No Change |
| Name: | Dr. Agata Exner |
| Project Role: | Co-PI (CWRU) |
| person month per year | No Change |
| Name: | Dr. Mithun Sinha |
| Project Role: | Co-I (IU) |
| person month per year | No Change |
| Name: | Dr. Atul Rawat |
| Project Role: | Postdoctoral Fellow (IU) |
| person month per year | No Change |
| Name: | Dr. Eric Abenojar |
| Project Role: | Post Doctoral Fellow (CWRU) |

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
    - Nothing to Report.
- **What other organizations were involved as partners?**
    - **Organization Name:** Case Western Reserve University
    - **Location of Organization:** Domestic
    - **Partner's contribution to the projec**t:
        - **Dr. Agata Exner** is co-PI (CWRU site PI), she has partnered in the NB fabrication and optimizations as described under Task 1.

**ADDITIONAL NOTES:**

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