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TITLE: Targeted Nanobubble Technology to Control Diabetic Macrophage Function

PRINCIPAL INVESTIGATOR: Dr. Sashwati Roy

CONTRACTING ORGANIZATION:
Indiana University, Indianapolis

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

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Indianapolis, IN 46202-5130

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**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. In preliminary experiments, the delivery resulted in improved wound closure in a delayed wound healing mice model of miR-21lysM cre.

**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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# 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 (NBmϕ-miR-21).

Specific Aim 2-Test whether NBmϕ-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 | ongoing | Partial complete |
| **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 | Partial complete |
| Subtask 2.2 *Confirm if delivery miR-21 to wound-site macrophages improves macrophage plasticity and facilitates resolution of wound inflammation healing.* | 6-18 | ongoing | Partial 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 | Partial complete |

- **What was accomplished under these goals?**

  - *For this reporting period describe:*

**1) major activities.** Towards major task1, we developed macrophage targeting gas nanobubbles (NB) carrying miR-21 mimic cargo. This included preparation, characterization and optimization platform cationic NBs which are needed to carry the miR-21 cargo, 2) modified the shell of the NBs with mannose to serve as a specific macrophage targeting moiety, and 3) loaded the NBs to FAM DNA as a fluorescent mimic of genetic material for wound healing experiments proposed in Task 2.

Major Task 2, our primary focused in optimizing delivery and tracking of miR-21 to diabetic wound-site macrophages in an excisional wound model (subtask 2.1) and conducted initial 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**

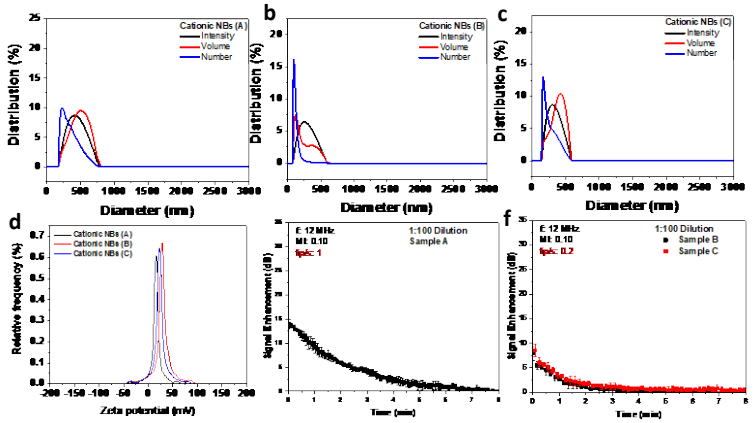


**Figure 1**. (a-c) DLS size measurement of bubbles for cationic NB samples A, B, and C, (d) comparison of the zeta potential measurement for the three samples showing that they are all positively charged, and (e,f) Stability of the samples under ultrasound exposure was performed and the corresponding signal intensity vs time curves are shown for the different bubble samples. Notice that at the same dilution and even with a lower frame rate the ultrasound signal enhancement for samples A and B is much lower and decay much faster compared to sample A.

**Major Task 1 develop macrophage targeting gas nanobubbles carrying miR-21 mimic cargo.**

**[Proprietary Data]** *I. Optimization of cationic NB formulation:* Three formulations were developed (Table 1) to observe the variation in size, charge, and stability under ultrasound exposure. With an increase in the cationic phospholipid, DOTAP, bubble size decreased and the positive charge (zeta potential) of the bubbles increased (**Figure 1 a-d**). An increase in bubble charge, however, led to destabilization of the NBs under ultrasound (**Figure 1 e and f**). Despite being exposed at a higher frame rate (5x faster), Sample A is still more stable over time under ultrasound compared to B and C at similar dilutions. Thus, we decided to use Sample A formulation moving forward.

**[Proprietary Data]** *II. Preparation of mannose-modified cationic NB:* Mannose-modified cationic NBs were characterized as above and show comparable results to Sample A indicating that the addition of PA-PEG3-mannose did not affect the physical properties of the material (**Figure 2**).

**[Proprietary Data]** *III. Preparation of mannose-modified cationic NB conjugated to FAM DNA*

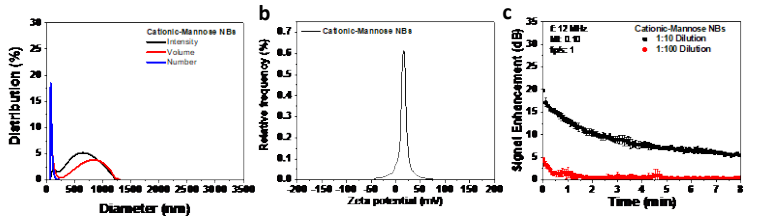


**Figure 2.** Mannose modified cationic NBs (mNB) were characterized with (a,b) DLS and zeta potential, and (c) signal stability over time under ultrasound exposure. At a lower dilution of 1:10 (and higher concentration) the bubbles are stable.

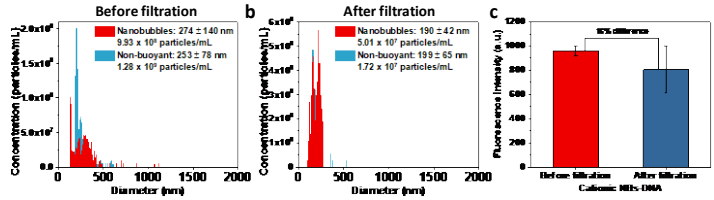


**Figure 3.** Mannose modified cationic NBs (mNB) before and after filtration were characterized with (a,b) RMM to determine mean size and concentration, and (c) fluorescence signal to determine DNA association.

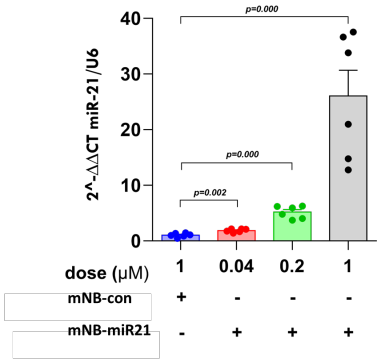


**Figure 4.** Optimization of dose dependent uptake of mNB-miR21 by human MDM. Human blood monocyte derived macrophages (MDM) were treated with either mannose modified NB containing miR-21 cargo (mNB-miR21) or mNB containing control (scrambled) cargo (mNB-con) for 24h. The cells were harvested and miR-21 abundance was determined using RTPCR. Data is mean±SD (n=6).

FAM DNA was used as an analog to evaluate whether the prepared bubbles can be conjugated to micro RNA. FAM DNA was added to NB solution after isolation and characterized before and after filtration, which was performed to remove excess/free FAM DNA in the samples that are not conjugated to the bubbles. RMM showed a decrease (an order of magnitude lower) in mean size after filtration as well as lower bubble concentration (**Figure 3 a,b**). However, filtration resulted in a higher buoyant (bubble) population in the sample. Fluorescent signal measurement before and after filtration showed a 16% decrease in signal, which may indicate the presence of free FAM DNA in the sample that were removed following filtration.

*IV) Uptake of mannose-modified cationic NB containing miR-21 cargo (mNB-miR21) by macrophages.* Human blood monocyte derived macrophages (MDM) were treated with either NB containing miR-21 cargo (mNB-miR21) or NB containing control (scrambled) cargo (mNB-con) for 24h. The cells were harvested and miR-21 abundance was determined using RTPCR (**Figure 4**). A dose dependent increase in miR-21 abundance indicate a successful delivery of miR-21 using mNB-miR21 as compared to the control mNB-con suggesting successful delivery of the cargo in MDM.

## 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

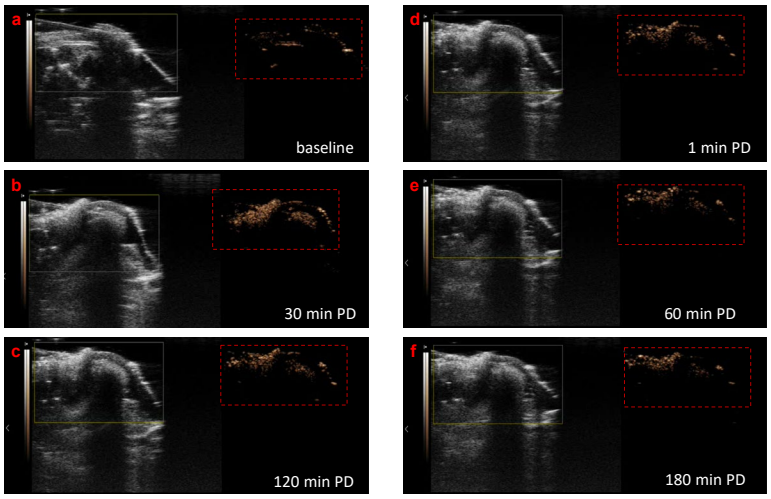


**Figure 5.** Topical delivery and tracking of mNB-Fam in excisional wounds. To optimize delivery of mNB-miR21 in excisional wounds, we created two punch biopsies (6 mm) at the dorsal side of mice. Splinting ring of silicone were sutured onto the wound to prevent healing by contraction. The mNB-Fam were injected at the wound edge via intradermal delivery. The mNB-Fam were tracked using ultrasound imaging (Vevo 2100 imaging system). Time lapse images have been shown. PD= post intradermal injection.

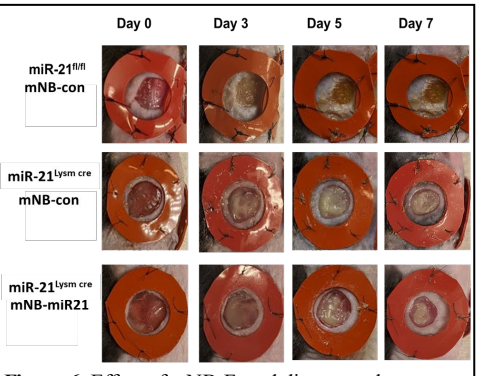


**Figure 6.** Effect of mNB-Fam delivery on the wound healing in mice with macrophage specific miR-21 knockdown (miR-21$^{lysM\ cre}$) of delayed wound healing mice model. Two punch biopsies (6 mm) at the dorsal side of mice were created followed by splinting to prevent healing by contraction. mNB-miR21 was delivered via intradermal injection as well as topically followed by imaging for 7 days to observe wound closure. Post day 7 wounds were harvested and miR-21 levels were determined in the wound tissue. Representative wound closure

**[Proprietary Data]** *V) Topical delivery and tracking of mNB-Fam in excisional wounds.* To optimize delivery of mNB-miR21 in excisional wounds, we created two punch biopsies (6 mm) at the dorsal side of mice. Splinting ring of silicone were sutured onto the wound to prevent healing by contraction. For tracking purposes, we utilized FAM -labelled mNB (mNB-Fam). The mNB-Fam was either delivered topically via intradermal injection. The nanobubbles were tracked on a real-time basis using Vevo 2100 imaging system. Time lapse images clearly indicate signals appears and strengthens by 30 min then the signal starts fading away (**Figure 5**).

**[Proprietary Data]** VI) Effect of mNB-Fam delivery on the wound healing in mice with macrophage specific miR-21 knockdown (miR-21$^{lysM\ cre}$) in mice. Because of Covid-19, all the mice deliveries were stopped, for this reason, we decided to test the effect of mNB-miR21 on wound healing was tested in a delayed wound healing model that was available in house. These preliminary experiments were conducted to determine if wound edge intradermal delivery results in any improvement in wound closure (**Figure 6**). Also, whether such delivery increases level of miR-21 in wounds was tested (data not shown). Preliminary data obtained indicate a beneficial effect of mNB-miR-21 delivery in wounds.

**4) other achievements**.

- We successfully formulated cationic nanobubbles which are sufficiently stable to allow for adequate therapy time. We also targeted these NBs to mannose and examined the effect of mannose on the stability of the nanobubbles. While the stability was decreased, the duration of signal is still sufficient for therapy. The US signal shows presence of gas in the bubble core. The gas is essential to the acoustic activity of the bubbles. Finally, we showed that we can load DNA onto the NBs and that following size exclusion chromatography, we nonetheless maintain a high level of NBs with cargo. Future work will focus on imaging and destroying the loaded-NBs with ultrasound and application of the mi-R21 cargo to the ultimate clinical problem.

- We also have optimized the delivery of mNB in excisional wounds that was trackable under US imaging using Vevo2100. In preliminary experiments, the delivery resulted in improved wound closure in a delayed wound healing mice model of miR-21$^{lysM\ cre}$.

    - **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 2021, WHS 2021.

        Dissemination of our findings and the newly developed treatment techniques to other military healthcare providers and researchers will be accomplished through presentations and in-service training at major meetings and conferences within the Department of Defense, VHA and the general public.

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

        *We continue to make progress for ultrasound mediated gene delivery and the diabetic wound inflammation resolution, macrophage plasticity and other tasks pending in Aims 1& 2.*

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.

- **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 obtain preclinical data with diabetic ulcers that appears promising with the preliminary findings, 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 some delays that we faced because of COVID-19 related closure of Research facilities. The diabetic mice delivery required for Aim 2 was affected. Because of the delay, we are requesting a 12 month 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:**

**MARKING OF PROPRIETARY INFORMATION:** Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the COR/GOR to obtain approval. **REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE.** It is the responsibility of the Principal Investigator to advise the COR/GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS. DO NOT USE WATERMARKS WHEN MARKING DOCUMENTS.**

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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 (NBmϕ-miR-21).

Specific Aim 2-Test whether NBmϕ-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 | ongoing | Partial complete |
| **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 | Partial complete |
| Subtask 2.2 *Confirm if delivery miR-21 to wound-site macrophages improves macrophage plasticity and facilitates resolution of wound inflammation healing.* | 6-18 | ongoing | Partial 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 | Partial complete |

- **What was accomplished under these goals?**

  - *For this reporting period describe:*

**1) major activities.** Towards major task1, we developed macrophage targeting gas nanobubbles (NB) carrying miR-21 mimic cargo. This included preparation, characterization and optimization platform cationic NBs which are needed to carry the miR-21 cargo, 2) modified the shell of the NBs with mannose to serve as a specific macrophage targeting moiety, and 3) loaded the NBs to FAM DNA as a fluorescent mimic of genetic material for wound healing experiments proposed in Task 2.

Major Task 2, our primary focused in optimizing delivery and tracking of miR-21 to diabetic wound-site macrophages in an excisional wound model (subtask 2.1) and conducted initial 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**

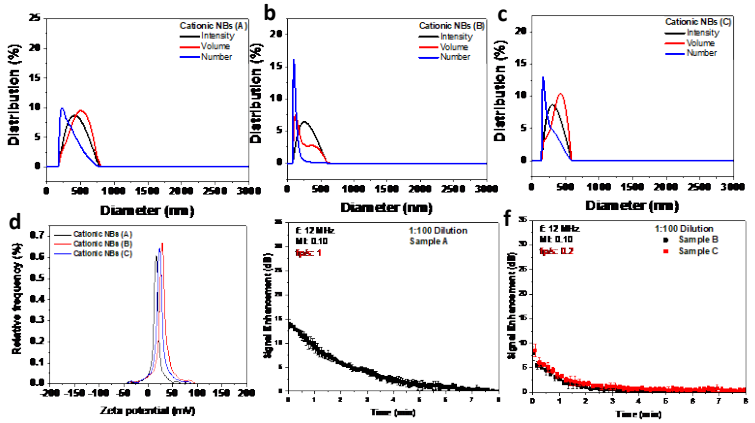


**Figure 1**. (a-c) DLS size measurement of bubbles for cationic NB samples A, B, and C, (d) comparison of the zeta potential measurement for the three samples showing that they are all positively charged, and (e,f) Stability of the samples under ultrasound exposure was performed and the corresponding signal intensity vs time curves are shown for the different bubble samples. Notice that at the same dilution and even with a lower frame rate the ultrasound signal enhancement for samples A and B is much lower and decay much faster compared to sample A.

**Major Task 1 develop macrophage targeting gas nanobubbles carrying miR-21 mimic cargo.**

**[Proprietary Data]** *I. Optimization of cationic NB formulation:* Three formulations were developed (Table 1) to observe the variation in size, charge, and stability under ultrasound exposure. With an increase in the cationic phospholipid, DOTAP, bubble size decreased and the positive charge (zeta potential) of the bubbles increased (**Figure 1 a-d**). An increase in bubble charge, however, led to destabilization of the NBs under ultrasound (**Figure 1 e and f**). Despite being exposed at a higher frame rate (5x faster), Sample A is still more stable over time under ultrasound compared to B and C at similar dilutions. Thus, we decided to use Sample A formulation moving forward.

**[Proprietary Data]** *II. Preparation of mannose-modified cationic NB:* Mannose-modified cationic NBs were characterized as above and show comparable results to Sample A indicating that the addition of PA-PEG3-mannose did not affect the physical properties of the material (**Figure 2**).

**[Proprietary Data]** *III. Preparation of mannose-modified cationic NB conjugated to FAM DNA*

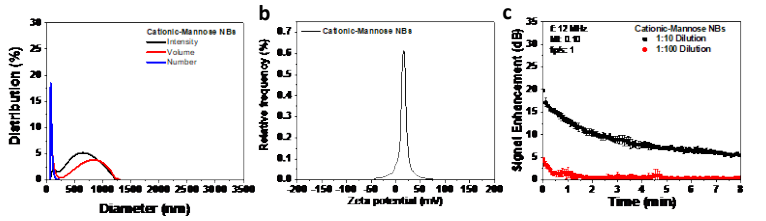


**Figure 2.** Mannose modified cationic NBs (mNB) were characterized with (a,b) DLS and zeta potential, and (c) signal stability over time under ultrasound exposure. At a lower dilution of 1:10 (and higher concentration) the bubbles are stable.

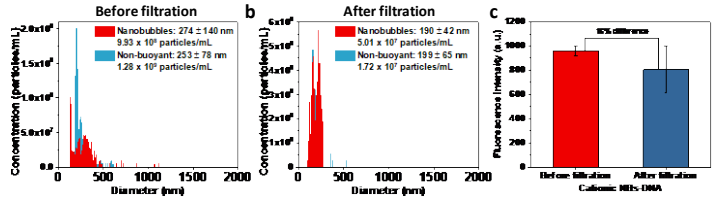


**Figure 3.** Mannose modified cationic NBs (mNB) before and after filtration were characterized with (a,b) RMM to determine mean size and concentration, and (c) fluorescence signal to determine DNA association.

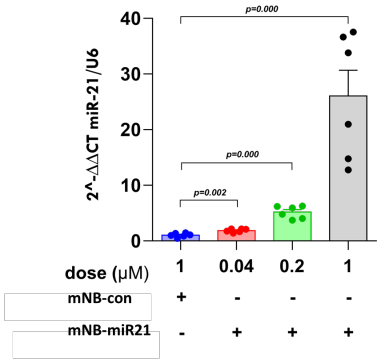


**Figure 4.** Optimization of dose dependent uptake of mNB-miR21 by human MDM. Human blood monocyte derived macrophages (MDM) were treated with either mannose modified NB containing miR-21 cargo (mNB-miR21) or mNB containing control (scrambled) cargo (mNB-con) for 24h. The cells were harvested and miR-21 abundance was determined using RTPCR. Data is mean±SD (n=6).

FAM DNA was used as an analog to evaluate whether the prepared bubbles can be conjugated to micro RNA. FAM DNA was added to NB solution after isolation and characterized before and after filtration, which was performed to remove excess/free FAM DNA in the samples that are not conjugated to the bubbles. RMM showed a decrease (an order of magnitude lower) in mean size after filtration as well as lower bubble concentration (**Figure 3 a,b**). However, filtration resulted in a higher buoyant (bubble) population in the sample. Fluorescent signal measurement before and after filtration showed a 16% decrease in signal, which may indicate the presence of free FAM DNA in the sample that were removed following filtration.

*IV) Uptake of mannose-modified cationic NB containing miR-21 cargo (mNB-miR21) by macrophages.* Human blood monocyte derived macrophages (MDM) were treated with either NB containing miR-21 cargo (mNB-miR21) or NB containing control (scrambled) cargo (mNB-con) for 24h. The cells were harvested and miR-21 abundance was determined using RTPCR (**Figure 4**). A dose dependent increase in miR-21 abundance indicate a successful delivery of miR-21 using mNB-miR21 as compared to the control mNB-con suggesting successful delivery of the cargo in MDM.

## 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

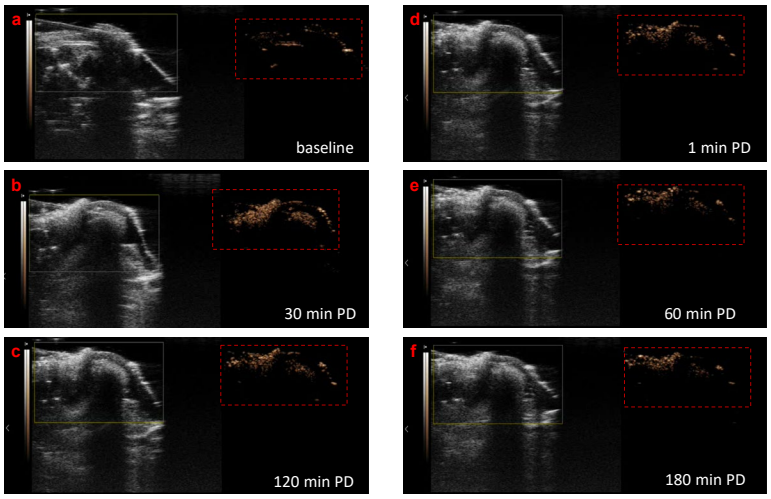


**Figure 5.** Topical delivery and tracking of mNB-Fam in excisional wounds. To optimize delivery of mNB-miR21 in excisional wounds, we created two punch biopsies (6 mm) at the dorsal side of mice. Splinting ring of silicone were sutured onto the wound to prevent healing by contraction. The mNB-Fam were injected at the wound edge via intradermal delivery. The mNB-Fam were tracked using ultrasound imaging (Vevo 2100 imaging system). Time lapse images have been shown. PD= post intradermal injection.

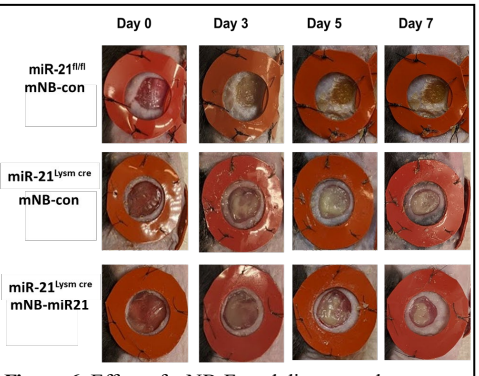


**Figure 6.** Effect of mNB-Fam delivery on the wound healing in mice with macrophage specific miR-21 knockdown (miR-21$^{lysM\ cre}$) of delayed wound healing mice model. Two punch biopsies (6 mm) at the dorsal side of mice were created followed by splinting to prevent healing by contraction. mNB-miR21 was delivered via intradermal injection as well as topically followed by imaging for 7 days to observe wound closure. Post day 7 wounds were harvested and miR-21 levels were determined in the wound tissue. Representative wound closure

**[Proprietary Data]** *V) Topical delivery and tracking of mNB-Fam in excisional wounds.* To optimize delivery of mNB-miR21 in excisional wounds, we created two punch biopsies (6 mm) at the dorsal side of mice. Splinting ring of silicone were sutured onto the wound to prevent healing by contraction. For tracking purposes, we utilized FAM -labelled mNB (mNB-Fam). The mNB-Fam was either delivered topically via intradermal injection. The nanobubbles were tracked on a real-time basis using Vevo 2100 imaging system. Time lapse images clearly indicate signals appears and strengthens by 30 min then the signal starts fading away (**Figure 5**).

**[Proprietary Data]** VI) Effect of mNB-Fam delivery on the wound healing in mice with macrophage specific miR-21 knockdown (miR-21$^{lysM\ cre}$) in mice. Because of Covid-19, all the mice deliveries were stopped, for this reason, we decided to test the effect of mNB-miR21 on wound healing was tested in a delayed wound healing model that was available in house. These preliminary experiments were conducted to determine if wound edge intradermal delivery results in any improvement in wound closure (**Figure 6**). Also, whether such delivery increases level of miR-21 in wounds was tested (data not shown). Preliminary data obtained indicate a beneficial effect of mNB-miR-21 delivery in wounds.

**4) other achievements**.

- We successfully formulated cationic nanobubbles which are sufficiently stable to allow for adequate therapy time. We also targeted these NBs to mannose and examined the effect of mannose on the stability of the nanobubbles. While the stability was decreased, the duration of signal is still sufficient for therapy. The US signal shows presence of gas in the bubble core. The gas is essential to the acoustic activity of the bubbles. Finally, we showed that we can load DNA onto the NBs and that following size exclusion chromatography, we nonetheless maintain a high level of NBs with cargo. Future work will focus on imaging and destroying the loaded-NBs with ultrasound and application of the mi-R21 cargo to the ultimate clinical problem.

- We also have optimized the delivery of mNB in excisional wounds that was trackable under US imaging using Vevo2100. In preliminary experiments, the delivery resulted in improved wound closure in a delayed wound healing mice model of miR-21$^{lysM\ cre}$.

    - **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 2021, WHS 2021.

        Dissemination of our findings and the newly developed treatment techniques to other military healthcare providers and researchers will be accomplished through presentations and in-service training at major meetings and conferences within the Department of Defense, VHA and the general public.

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

        *We continue to make progress for ultrasound mediated gene delivery and the diabetic wound inflammation resolution, macrophage plasticity and other tasks pending in Aims 1& 2.*

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.

- **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 obtain preclinical data with diabetic ulcers that appears promising with the preliminary findings, 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 some delays that we faced because of COVID-19 related closure of Research facilities. The diabetic mice delivery required for Aim 2 was affected. Because of the delay, we are requesting a 12 month 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:**

**MARKING OF PROPRIETARY INFORMATION:** Data that was developed partially or exclusively at private expense shall be marked as "Proprietary Data" and Distribution Statement B included on the cover page of the report. Federal government approval is required before including Distribution Statement B. The recipient/PI shall coordinate with the COR/GOR to obtain approval. **REPORTS NOT PROPERLY MARKED FOR LIMITATION WILL BE DISTRIBUTED AS APPROVED FOR PUBLIC RELEASE.** It is the responsibility of the Principal Investigator to advise the COR/GOR when restricted limitation assigned to a document can be downgraded to "Approved for Public Release." **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS. DO NOT USE WATERMARKS WHEN MARKING DOCUMENTS.**