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TITLE: Development of a novel platform for in vivo delivery of antagomirs to study cetuximab resistance in colorectal cancer

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14. ABSTRACT

The subject of the research is colorectal cancer treatment, and more specifically, overcoming epigenetic mechanisms of acquired cetuximab resistance in colorectal cancer. We have identified the epigenetic mechanisms that lead to cetuximab resistance in many patients, but the targets are microRNA molecules that are not able to be targeted by traditional drugs. The purpose of our research is to develop a novel delivery mechanism for so-called antagomirs, which could reverse cetuximab resistance. The scope of the proposed work will develop a novel delivery platform and to ultimately test in animal models cetuximab-resistant colorectal cancer to determine if it is efficacious. To date we have made significant progress on the synthesis of our antagomir delivery molecules, though that progress has been hampered in part by COVID-related absence from the laboratory.

15. SUBJECT TERMS

Colorectal cancer, PET imaging, molecular imaging, microRNA, cetuximab, cetuximab resistance, antagomir, precision medicine, epigenetics

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INTRODUCTION:

The subject of the research is colorectal cancer treatment, and more specifically, overcoming epigenetic mechanisms of acquired cetuximab resistance in colorectal cancer. We have identified the epigenetic mechanisms that lead to cetuximab resistance in many patients, but the targets are microRNA molecules that are not able to be targeted by traditional drugs. The purpose of our research is to develop a novel delivery mechanism for so-called antagomirs, which could reverse cetuximab resistance. The scope of the proposed work will develop a novel delivery platform and to ultimately test in animal models cetuximab-resistant colorectal cancer to determine if it is efficacious.

1. KEYWORDS:

Colorectal cancer, PET imaging, molecular imaging, microRNA, cetuximab, cetuximab resistance, antagomir, precision medicine, epigenetics

2. ACCOMPLISHMENTS:

What were the major goals of the project?

This project began in August 2019 and work in our laboratory ceased due to the COVID-19 pandemic in March 2020. After our laboratory moved to Stony Brook University (SBU), the grant was transferred and work resumed in November of 2020, albeit at a reduced pace due to COVID-based restrictions on lab personnel lasting through April 2021.

The overall goal of this project is to develop a platform for delivering labeled antagomirs to human colorectal cancer (CRC) cells utilizing an affibody targeting vector and a linear polyethylenimine (LPEI) delivery vector. Before in vitro and in vivo experiments can take place, we first have to produce the triconjugate polyplex. The high cationic charge density of the LPEI forms a non-covalent complex with the antagomir known as a polyplex, which is then internalized by cells resulting in delivery of the nucleic acid inside the cell. Specificity in this delivery is obtained by covalently linking the polyplex to an affibody targeting vector via a polyethylene glycol (PEG) phenyloxadiazole (PODS) based linker. The PEG part of this linker reacts with one of the secondary amines on the LPEI via a terminal N-hydroxysuccinimide (NHS) ester moiety at the end of the PEG to form the diconjugate (Fig 1, step 1). The PODS is then attached via dibenzocyclooctyne (DBCO) based copper free click chemistry (Fig 1, step 2). The PODS then reacts specifically with a terminal cystine engineered into the affibody to form the triconjugate (Fig 1, step 3). The major goals of our project through this time frame were the “Synthesis of 1:1 and 1:3 triconjugates” as outlined in our SOW. To date, we have completed ~90% of this work.

What was accomplished under these goals?

Major Activities and Specific Objectives

We proposed to improve upon previous iterations of polyplexes by utilizing a new linker strategy that is based on work recently reported by the Zeglis group in Bioconjugate Chemistry. The previous syntheses of similar polyplexes utilized an orthopyridyl disulfide (OPSS) moiety to directly conjugate with the cystine tag on the affibody. However, the disulfide bond formed in this conjugation is not stable in vivo, so we sought to utilize a phenyloxadiazolyl methylsulfone (PODS) based conjugation because it has been shown to exhibit remarkable stability in vivo. Our main objective at this point in our research was to develop synthesis, purification, and characterization methods for the "triconjugates" based on our novel approach, which would be the result of step 3 in Figure 1.

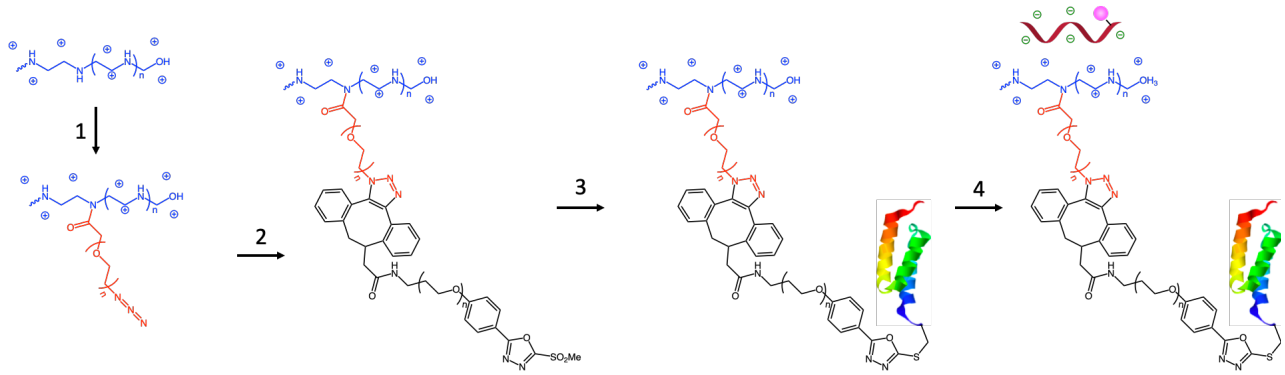

Figure 1: Proposed Synthesis. First, the PEG-N_3 conjugated to the LPEI via NHS ester reaction with one of the secondary amines on the LPEI. Then the PODS is attached via DBCO copper free click chemistry. Then the affibody is attached by a reaction between the PODS and a terminal cystine engineered into the affibody. Finally, the antagomir is added via a non-covalent complexation with the LPEI. The final result is an affibody-LPEI-antigomir triconjugate polyplex.

Before the first step in the synthesis could be completed, it was necessary to setup a system for analysis and purification of the products. This proved to be more challenging than initially anticipated. The previous work in the literature utilized an advanced fast protein liquid chromatography (FPLC) unit and methodolgy based on ion exchange chromatography (IEC).

Previous work was done in the Houghton Lab on a ÄKTA Start FPLC at VUMC, however a new system was purchased from BioRad to carry out the remainder of the work. A significant amount of work developing methods for the new system was required. This work was done during the reporting time period with good results. The lab is now able to use the new system to separate the LPEI products after synthesis. In addition to the ion exchange method in reported in the literature, the Houghton lab has developed a method using size exclusion chromatography (SEC) to perform the separations without the need to vary the salt content of the eluent (**Figure 2**). This has eliminated the need for a dialysis step in the purification to remove excess salt. Via our new methodology, we have confirmed that previously reported synthetic scheme did not produce the well characterized mono- and tri-conjugates, and this will be discussed in our manuscripts.

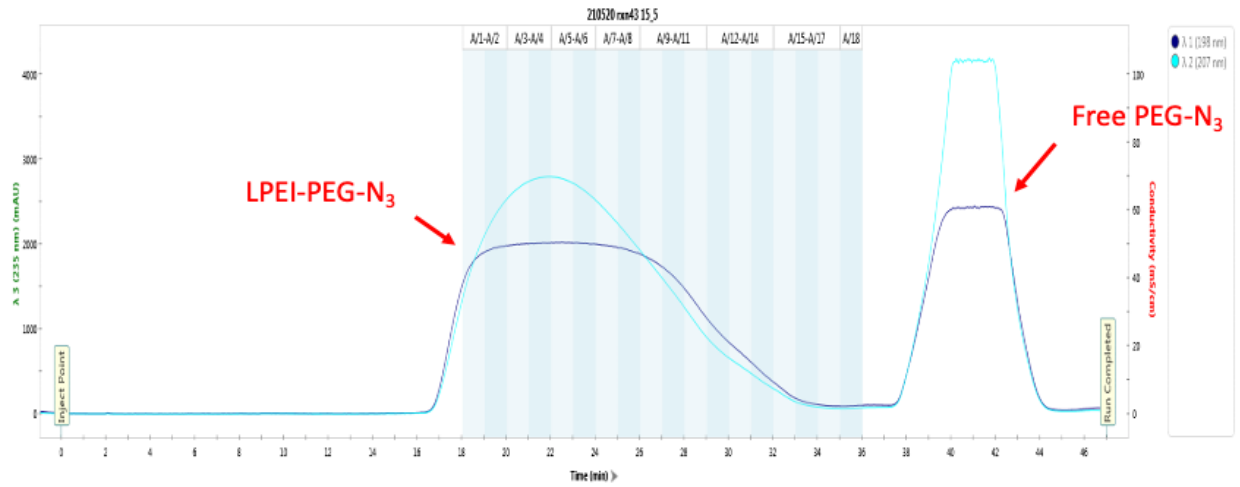

Figure 2: SEC Method Development. This SEC chromatogram shows the results typical purification of the first step of a reaction. Here, the LPEI hydrochloride salt that is commercially available was first converted to the base, then reacted with 5eqs of PEG at room temperature for 3 hours, then converted back into the hydrochloride salt before being separated from the unreacted PEG on a Cytiva Superdex 75 GL SEC column.

In the first step of the triconjugate synthesis, we sought to incorporate an azide (N_3) functionalized PEG into the linear polyethylenimine (LPEI) backbone, rather than previously utilized OPSS functionalized PEG in the first step. During this time period, we worked to optimize this synthesis with good results. We are now able to tune the ratio of the ethylene glycol moieties (EG) to the ethylenimine (EI) moieties (**Figure 3**). This method is more complex but also much more precise than previously reported methods, and we believe represents a significant advancement in the production of these polymer-based oligonucleotide deliver vehicles.

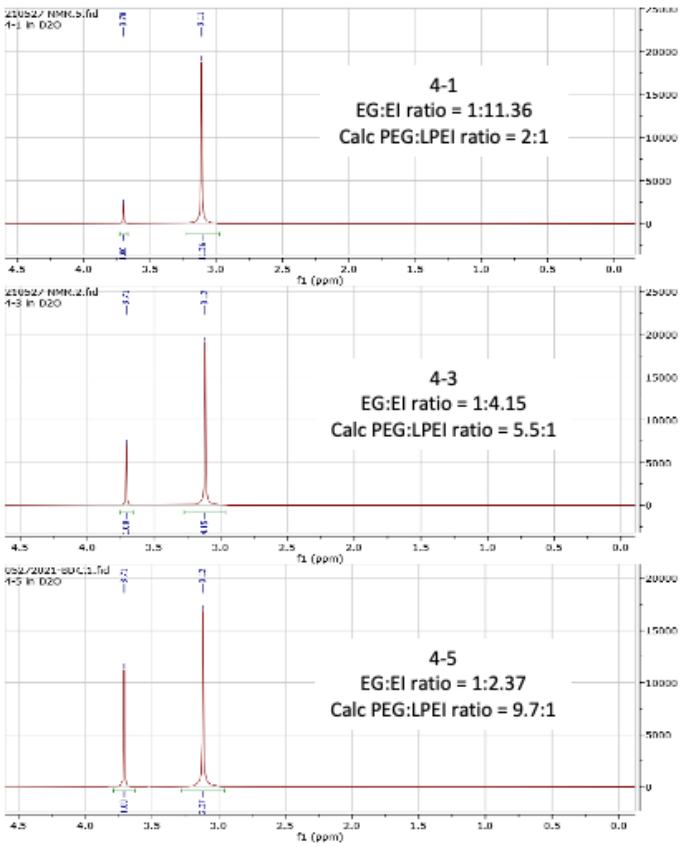

Figure 3: Tuning the EG:EI ratio. These NMRs show differing EG:EI ratios based solely on the amount of PEG added (1, 3, and 5 eqs). All syntheses were done at similar scales (~20mg) and with similar reaction conditions (3 hours at room temp).

In addition to tuning the EG:EI ratio, we also developed a method to use SEC chromatography to separate different length LPEI polymers. We found that using different length LPEI chains could affect the EG:EI ratio up to 40% (**Figure 4**).

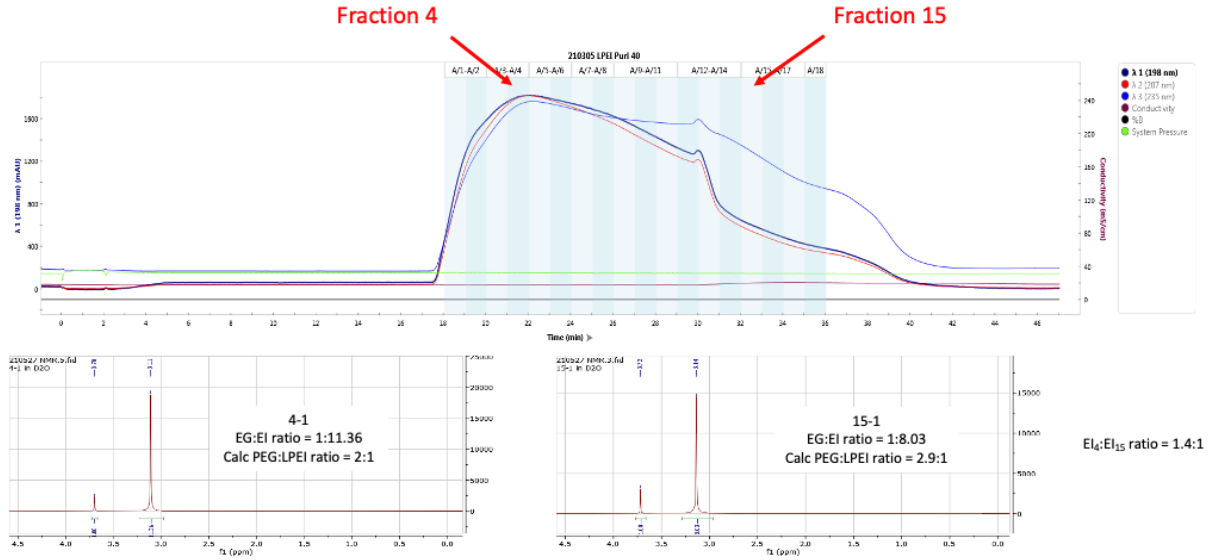

Figure 4: SEC separation of LPEI. The top chromatogram shows the separation of different length LPEI chains via SEC chromatography. The NMRs show differing EG:EI ratios based solely on the length of LPEI chain. Fraction 4 is the longer chain and fraction 15 is the shorter chain. Both syntheses were done at similar scales (~20mg) and with similar reaction conditions (1eq PEG, 3 hours at room temp).

Simultaneously to work on the LPEI-PEG-N_3, we have also worked to produce and validate the novel site specific linker PODS-DBCO (**Figure 5**). Previously, we have developed the synthesis and characterized the molecule. During this reporting period, we have worked to produce an amount of the linker adequate for the remainder of the study and have also validated the linker with some test conjugations with the affibody. We are currently waiting for delivery of a new gel filtration chromatography column that will allow us to separate and quantify the conjugated components, which we expect this month. The next step will be to assemble the full polyplex and begin screening conditions for complexation with the antagomirs that are scheduled to be delivered in October 2021.

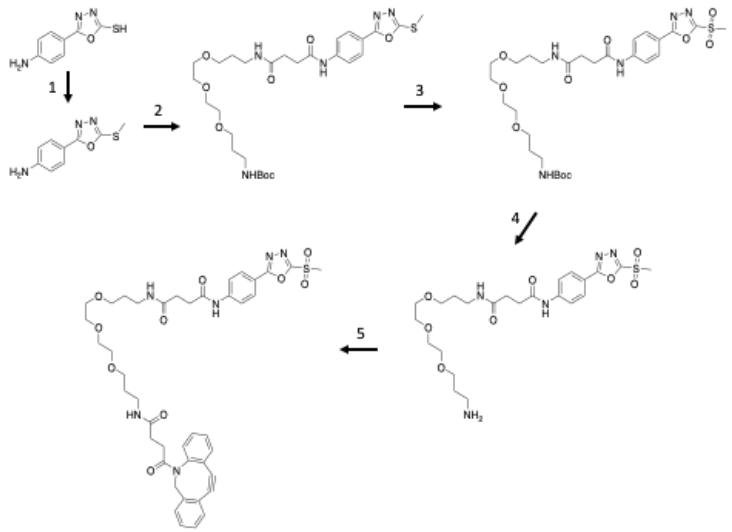

Figure 4: PODS-DBCO Synthetic Scheme. First the thiol group is methylated with iodomethane (1), then the PEG group is added with NHS ester chemistry (2). The sulfur is then oxidized with mCPBA (3) and the primary amine is deprotected (4). Finally, the DBCO is added with NHS ester chemistry to yield the final product (5).

COVID-related shut downs and limitations to lab access have made the past period difficult. Additional vendor-related delays have also made progress beyond the current point impossible, as we had extensive delays in equipment installation (e.g. Biorad purification system), delivery of our TOPFlash kits, and delivery of our antagomirs. Despite these temporary delays, we have made phenomenal progress on the novel chemistry, purification, and characterization methods for the polyplexes and the delays seem to be abating, so we are excited to continue the project in the coming year. We do anticipate submitting a request for a no cost extension before the end of the year, so that we may continue this project now that our laboratory is fully functional and accessible to all staff and trainees.

Significant results

- Polyplex synthesis
    - Developed and optimized purification techniques LPEI-PEG-N_3
        - Dr. Carney trained in SEC method development
    - Optimized synthesis conditions for LPEI-PEG-N_3
    - Developed techniques for tuning EG:EI ratios
    - Produced and validated PODS-DBCO
    - PODS-DBCO conjugated to affibody
        - Optimization currently underway
    - New antagomirs ordered – estimated arrival in October 2021

- Cell lines
    - All cell lines obtained, cultured, and stocks frozen
    - Plating density and growth conditions completed for each cell line
    - CC-CR transfection with TOPFlash underway – actively screening conditions

- IACUC and ACURO approval obtained at SBU
    - Animal studies may commence when polyplexes completed

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

Training

Dr. Carney began training in polymer and bioconjugation chemistry with Dr. Houghton in August 2019 and began working on this project in January 2020. In addition to learning chemical synthesis techniques, he also learned how to perform numerous purification techniques on poly ionic compounds using ion exchange chromatography. Dr. Carney is an accomplished radiochemist but these techniques and principles were new to him at the time and he has exceeded expectations.

Dr. Carney has continued to develop his background in polymer synthesis and bioconjugation chemistry during the past award period. While it was a difficult transition from VUMC to SBU due to the COVID-19 pandemic, Dr. Carney has been very productive, especially considering the circumstances. He has been instrumental in researching new instrumentation and setting up said instrumentation in our new labs at SBU, which has been a great learning experience for him. He has gained extensive expertise with ion exchange chromatography, gel filtration chromatography, bioconjugation chemistry, polymer chemistry, polymer characterization. He also has expanded his knowledge of colorectal cancer biology and will focus on furthering that expertise in the coming year now that he has developed the technical expertise to carry out all of the chemistry and basic biological assays required for the project.

Dr. Houghton has continued to gain didactic training in colorectal cancer biology and imaging by attending the weekly Coffey Group Meeting, the Epithelial Biology Center (EBC) monthly seminars, and Vanderbilt Digestive Disease Resource Center (VDDRC) seminar series. Fortuitously, these meetings became virtual shortly after the lab moved to SBU, which has allowed him to continue to actively participate beyond what may have been possible prior to the pandemic. Dr. Coffey's research as well as that of the researchers in the EBC and VDDRC are largely focused on colorectal cancer. Attending these meetings and seminars have allowed Dr. Houghton to expand upon his knowledge base in colorectal cancer. These training opportunities are in line with the proposed Career Development tasks. Dr. Houghton and Dr. Coffey have continued planning for potential grant applications (e.g. Impact Award) for research that we anticipate we result directly from this project. While slated to take place during year 2, the pandemic and move from VUMC to SBU have slightly delayed the anticipated date of submission for these applications, but this setback has been completely offset by Dr. Houghton and his lab being given the appropriate resources and lab space to carry out the proposed work. During the pandemic-related work from home order, we turned our efforts into an exhaustive analysis of the literature and plans for future grants.

Professional Development

Dr. Houghton has continued to work with Dr. Coffey to expand his network of GI and colorectal cancer experts during this period of the award. Additional opportunities have been afforded to Dr. Houghton since the move to SBU including participation in numerous groups that focus on imaging and cancer biology including, becoming a member and regularly presenting to the Imaging, Biomarker Discovery, and Engineering Sciences program, participating in the pancreatic and colorectal CRTs, and presenting for Pathology Grand Rounds. Additionally, Dr. Houghton has begun the process of becoming a full affiliate member of the Chemistry Department at SBU where he will be able to recruit students to continue this project long term.

Shortly prior to arriving at SBU, I also became a member of the ad-hoc group of GI Cancer researchers. Our administration was aware of my DoD Career Development Award, and asked me to take part in this group that is working to develop program level grants in GI cancer. My project was identified as one that may be included in future grant applications, which continues to lead to great networking opportunities within my new institution. In sum, I would summarize my professional development as having benefitted from the recent challenging circumstances, including research delays due to COVID-19 and moving our laboratory.

Additionally, Dr. Carney has has great professional development opportunities as well. He has spent a majority of his time outside of the lab learning about colorectal cancer biology. He has done so be reading primary literature as well as attending Dr. Coffey's laboratory meetings and the Epithelial Biology Center seminars. During his short involvement in this project, he has made great strides in his understanding of colorectal cancer, microRNA signaling, and strategies for targeting microRNA-related epigenetic mechanisms of drug resistance. He has also networked extensively with the GI Cancer researchers at VUMC and will continue to do so at Stony Brook.

How were the results disseminated to communities of interest?

Nothing to report.

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

The non-covalent complexation of the antagomir to form polyplexes is performed after we have achieved the scale up of the triconjugates. This process is shown in Figure 1 (step 4) affibody-LPEI-antigomir triconjugate polyplex (Fig 1, step 4). A possible secondary strategy is to form the LPEI-PEG-N_3 and DBCO-PODS-Affibody constructs separately and then combine those two components. Based on further examination of literature precedent, we believe this strategy may be more feasible. While awaiting award transfer, we will investigate this alternative strategy.

4. **IMPACT:**

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

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

After outfitting the laboratory at SBU with new equipment that is suitable for the project we were able to confirm that the published methodology for synthesizing and purifying intermediates for the polyplexes were not reproducible under any circumstance. We conferred with numerous experts in polymer chemistry, and they confirmed that the published methods were ambiguous. We sought to identify alternative methods once arriving at SBU and installing the appropriate equipment for these studies. Dr. Carney has done a phenomenal job of developing a novel approach to prepare well-characterized polyplexes, which is reported above. The deviations from the plan were minor in scope but produced great results.

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

COVID-19 continued to cause significant delays on a number of fronts. Upon arriving at SBU, we were limited in our access to the laboratories, though those restrictions have been (at least temporarily) lifted as of this summer. Additionally, many vendors of critical instrumentation were not yet back to production and when they restarted they were forced to work through a back log of orders. Thus, our orders for FPLC was delayed by about 4 months. However, that instrument has been installed and has been used to acquire the reported data. Additionally, the TOPFlash reported kit required for our experiments was backordered for nearly 9 months due to the company being out of the lab due to the pandemic. Excitingly, we finally received the kit at the beginning of September and have prioritized the development of our TOPFlash harboring CC-CR cell lines. Additionally, the company from which we originally planned to source the antagomirs was acquired and *drastically* increased their prices. We have identified a new vendor and the antogamirs are expected to arrive in October 2021.

Changes that had a significant impact on expenditures

The restrictions on travel will lead to decreased expenditures on travel. Currently, we have not charged any travel to the award, and it seems unlikely any travel will be advisable in the coming year. Rather, we will utilize the travel funds to register for virtual meetings at a reduced price. Additionally, we have not spent as much as anticipated on radioactive isotopes or consumables during the first year. This is predominantly due to the change in institution as well as the reduced research performed due to COVID-related restrictions on laboratory access. We do not anticipate any impact on total expenditure.

Significant changes in use or care of human subjects

Does not apply.

Significant changes in use or care of vertebrate animals

We have not reached the point of animal studies at this point. We have received both institutional IACUC approval and ACURO approval and will be ready to move forward as soon as possible.

Significant changes in use of biohazards and/or select agents

No changes. All trainings and approvals at SBU are now completed.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

The work has yet to advance to the point of publishing.

Books or other non-periodical, one-time publications.

None.

Other publications, conference papers and presentations.

None.

- **Website(s) or other Internet site(s)**

None.

- **Technologies or techniques**

We have developed novel synthesis and purification strategies which will be published in a peer reviewed journal along with the rest of our study results. Our methodologies, unlike currently published methodologies will be easily reproducible from our published protocols which will allow any researchers to take advantage of these advances.

- **Inventions, patent applications, and/or licenses**

None

- **Other Products**

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Jacob Houghton, PhD
Project Role: PI
Nearest person month worked: 5.5
Contribution to Project: Dr. Houghton is the PI. His laboratory work to date has involved training Dr. Carney in the areas of polymer synthesis, purification, and characterization. Additionally, his work has made significant strides in his career development under the mentorship of Dr. Coffey (Career Guide). Dr. Houghton has regularly attended lab meetings in person and virtually when in person attendance was not possible as well as the Epithelial Biology Center monthly meetings. Dr. Houghton has also advanced his network significantly by pursuing a better environment for his laboratory to perform the proposed studies. During the pandemic-related work from home mandate, Dr. Coffey recommended that, as part of his career development efforts, Dr. Houghton work with Dr. Carney to thoroughly review relevant research literature with the aim of preparing applications for additional funding for research that will directly derive from the funded project.
Funding Support: All work on this project was supported by this award.

Name: Brandon Carney, PhD
Project Role: Postdoctoral Fellow
Nearest person month worked: 4.5
Contribution to Project: Dr. Carney's laboratory work to date has involved being trained in the areas of polymer synthesis, purification, and characterization. Dr. Carney is an excellent radiochemist but he has limited experience in polymer chemistry and characterization. He has been responsible for troubleshooting the methodology for purification and characterization of the polyplexes. During January – early March, Dr. Carney worked out new methodology for our equipment to successfully isolate the polyplex intermediates. During the work from home mandate and up to our departure from VUMC, Dr. Carney worked diligently to support Dr. Houghton's efforts to plan for future funding applications that are anticipated to derive directly from this research.
Funding Support: All work on this project was supported by this award.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Upon moving to Stony Brook University, Dr. Houghton was provided with start up funds. These funds are being utilized to outfit new laboratory space and to support staff and trainee salaries. This support will ensure that the Houghton Laboratory has all of the equipment and personnel required to complete the proposed studies. These funds are also being used to bridge the gap in funding while awaiting for award transfer. The new equipment and lab space will meet or exceed all requirements to complete the proposed studies.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: