AWARD NUMBER: W81XWH-19-1-0446

TITLE: Development of a Novel Platform for In Vivo Delivery of Antagomirs to Study Cetuximab Resistance in Colorectal Cancer

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REPORT DATE: January 2024

TYPE OF REPORT: FINAL

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
January 2024	Final	15Aug2019-30Sep2023

4. TITLE AND SUBTITLE
Development of a Novel Platform for In Vivo Delivery of Antagomirs to Study Cetuximab Resistance in Colorectal Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-19-1-0446

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)

Stony Brook University
Lauterbur Dr.
Stony Brook, NY 11794-7263

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

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.

15. SUBJECT TERMS
Colorectal cancer, PET imaging, molecular imaging, microRNA, cetuximab, cetuximab resistance, antagomir, precision medicine, epigenetics

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRDC
a. REPORT	b. ABSTRACT	c. THIS PAGE	Unclassified	25	19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified			

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

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

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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 were the "Synthesis of 1:1 and 1:3 triconjugates", the development of a transiently transfected luciferase cell line to provide a read out of the biological effects of the antagomirs, and to test their ability to reverse cetuximab resistance in vitro and using in vivo mouse models of CRC.

What was accomplished under these goals?

Major Activities and Specific Objectives

Major Task 1 (partially completed)

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 first objective 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.

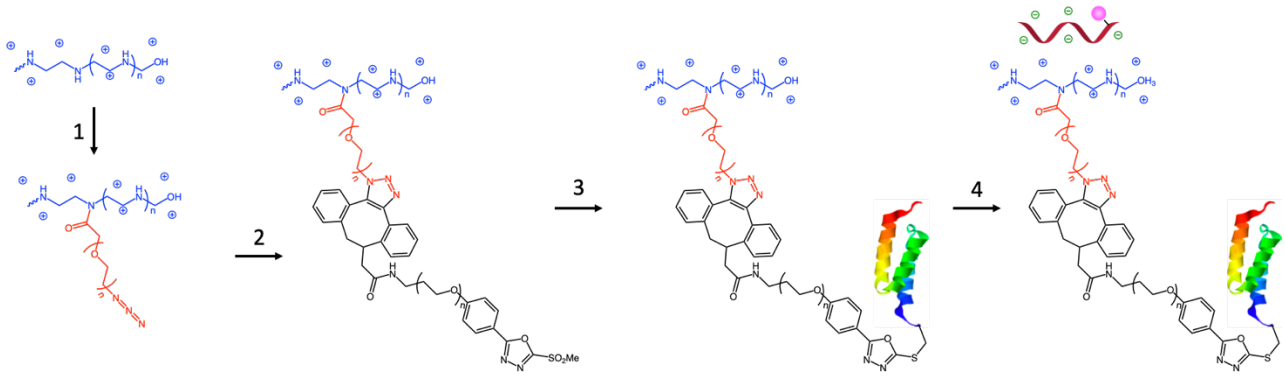

Figure 1: Proposed Synthesis. First, the PEG-N_3 conjugated to the LPEI via NHS ester reaction with one of the secondary amines on the LPEI. Then the PODS is attached via DBCO copper free click chemistry. Then the affibody is attached by a reaction between the PODS and a terminal cystine engineered into 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). Despite Dr. Houghton's start-up contract stating that all of his equipment needs would be met by the outfitting of a new floor and lab spaces, we were not allocated any funds to purchase the equipment necessary to achieve these research goals. Therefore, paid access time on an existing ÄKTA Start FPLC was provided by the Radiochemistry Core to perform these experiments. However, the ÄKTA Start was an inferior instrument and not capable of generating the same methods used in the literature, so a new methods had to be developed by Drs. Houghton and Carney. This method required many iterations to fully optimize so that it would be capable of separating LPEI conjugates from less densely charged precursors.

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. Surprisingly, the N_3-PEG has not exhibited the same reactivity as reported for the OPSS-PEG. Both PEG groups conjugate to the LPEI via NHS ester chemistry, and there is no reason to believe that the N_3 functional group should interfere in this reaction. However,

under the same reaction conditions reported for the OPSS-PEG (5 equivalents PEG stirring for 3 hours at room temperature in ethanol with the free base form of the LPEI), we observed no product (**Figure 2**). For that reaction, LPEI in the free base form was obtained by dissolving the hydrochloride salt obtained from Sigma into H2O (100mg in 5mL), then adding 6M NaOH until the final hydroxide concentration of the reaction mixture was 3M NaOH (5mL 6M NaOH added). The resulting precipitate was separated from the supernatant by centrifugation, washed 3 times with 1M NaOH, and then washed several times with deionized H2O until the wash tested neutral on pH paper. The resulting free base LPEI was dried overnight under vacuum, aliquoted and stored at -20°C for future experiments. In an example of the reaction based on literature precedence, 2mg of base form LPEI (LPEI_B) was dissolve in 100µL of EtOH. 0.5mg PEG-N_3 (5eq) in 100uL EtOH was added. The reaction was stirred for 3 hours at room temperature. The resulting LPEI was then converted back into its hydrochloride for by adding 400uL 1M HCl. The reaction was then air dried and redissolved in neutral H2O (pH of the solution was 7) before injection onto the FPLC.

The chromatogram in Figure 2 represents the UV absorbance of the components of the reaction mixture. The LPEI has no UV active functional groups, so passes through the detector without a signal. The only UV active group in the reaction mixture is the N_3 group, so the entire chromatogram represents the state of that group. The position of the arrow indicating the expected location LPEI-PEG-N_3 product is that of the product from subsequent experiments. This position also matches that of the LPEI-fluorescein conjugates used to optimize the FPLC method.

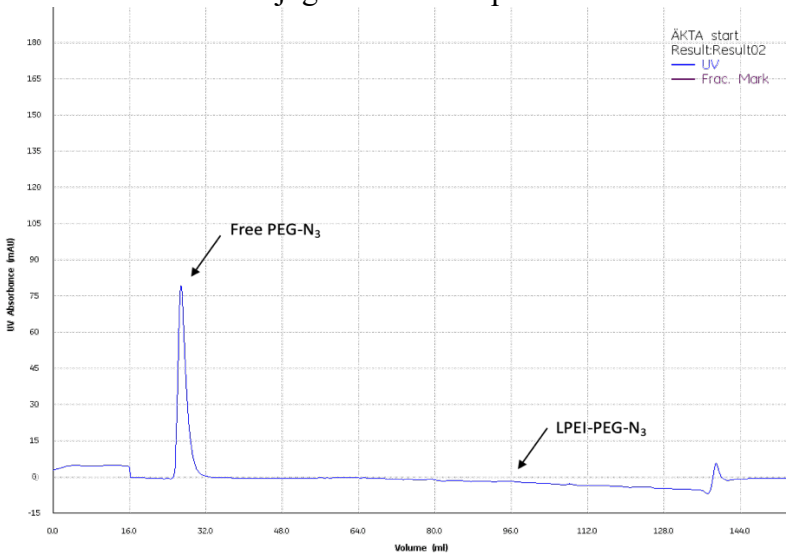

Figure 2: Literature Reaction. The results from the reaction using the procedure from the literature (at a smaller scale) showed no yield. This FPLC chromatogram shows the results of a reaction attempted using the procedure from the literature (at a smaller scale).

Further experiments showed a 5% yield utilizing N-butanol as a solvent and stirring for 3h at 100°C (**Figure 3**), however, the N_3 moiety is unstable at high temperatures for long periods of time. 2mg of base form LPEI (LPEI_B) was dissolve in 100µL of N-BuOH. 0.5mg PEG-N_3 (5eq) in 100uL N-BuOH was added. The reaction was stirred for 3 hours at 100°C. The resulting LPEI was then converted back into its hydrochloride for by adding 400uL 1M HCl. The reaction stirred for 30 minutes at room temperature and then air dried and redissolved in neutral H2O (pH of the solution

was 7) before injection onto the FPLC. Efforts leading up to the shutdown of all laboratory activities (February 2020) were focused upon scaling up this synthesis to acquire suitable amounts of material to proceed as no further increase in yield was observed at longer time points. *At this point in the development of the polyplexes, our laboratory at VUMC was shut down due to COVID-19 and prior to reopening out laboratory moved to Stony Brook Cancer Center. The impact of these closures and move are discussed in Section 5 of this report.*

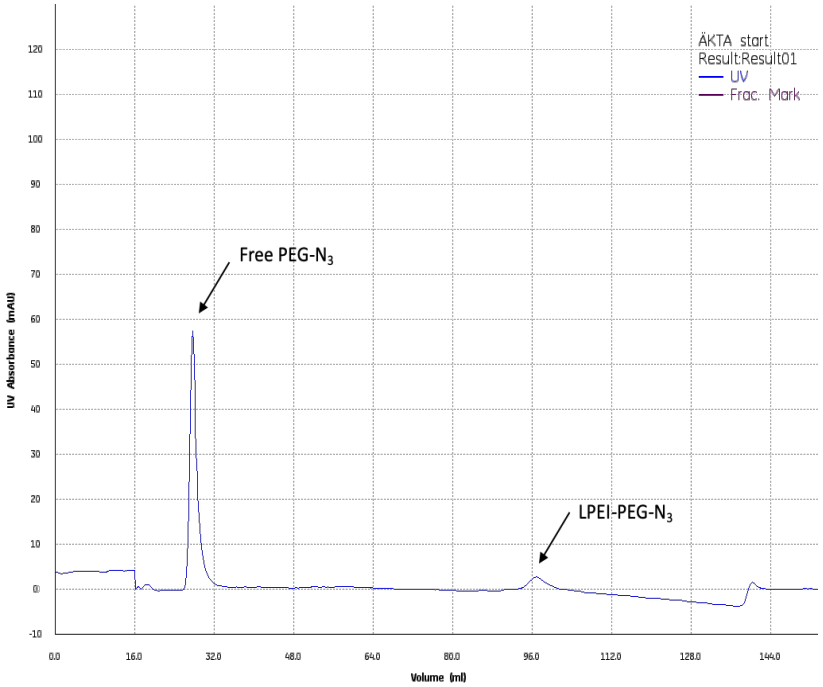

Figure 3: High Temperature Reaction. This FPLC chromatogram shows the results of a reaction attempted at much higher temperature using N-BuOH as a solvent to accommodate the higher temperature. 5% yield was determined by dividing the area of the LPEI-PEG-N_3 peak by that of the sum of the free PEG-N_3 and the LPEI-PEG-N_3 peaks adjusted for baseline.

Our laboratory moved to Stony Brook and this award transferred in September 2020, however we were not able to fully resume normal operations (with limited or no-COVID-19 related limitations) until early 2021. During the time we had limited access to the laboratory, we focused on setting up new instrumentation and preparing to resume normal laboratory functions to complete the project. 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 methodology based on ion exchange chromatography (IEC). Additionally, those methods, which reported a very specific ratio of PEG to LPEI, were not reproducible despite extensive efforts to optimize the reported procedure. We discussed this synthetic route with several nanomaterial experts at Vanderbilt University and Stony Brook University and concluded that the data reported was likely not reproducible as reported. Thus, we developed our own procedure and we successfully achieved formation of the triconjugates.

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 early to mid-2021 with good results which allowed us 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 developed a method using size exclusion chromatography (SEC) to perform the separations without the need to vary the salt content of the eluent (**Figure 4**). 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.

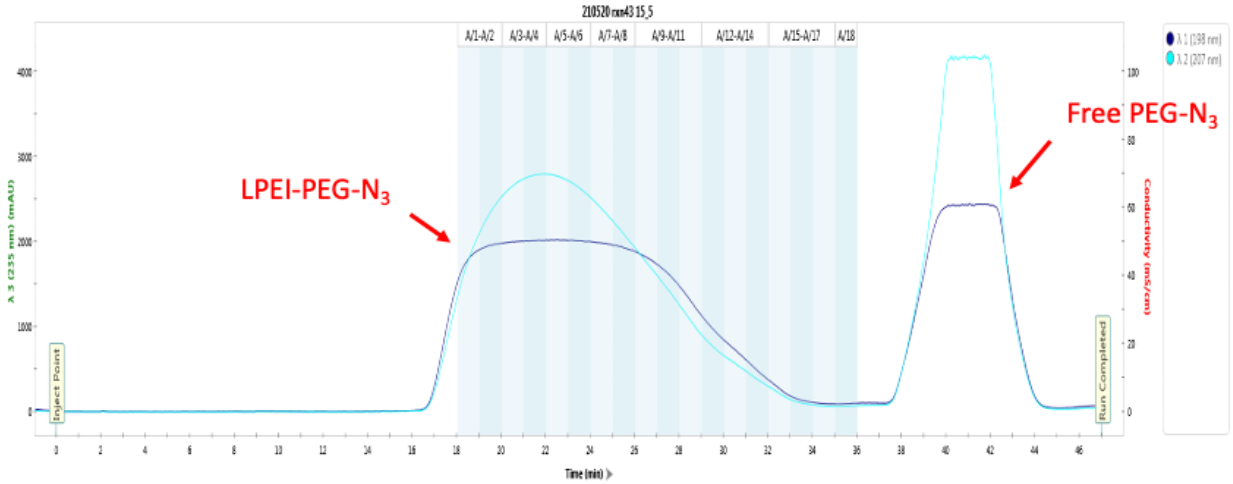

Figure 4: SEC Method Development. This SEC chromatogram shows the results typical purification of the first step of a reaction. Here, the LPEI hydrochloride salt that is commercially available was first converted to the base, then reacted with 5eqs of PEG at room temperature for 3 hours, then converted 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. Surprisingly, the N_3-PEG has not exhibited the same reactivity as reported for the OPSS-PEG. Both PEG groups conjugate to the LPEI via NHS ester chemistry, and there is no reason to believe that the N_3 functional group should interfere in this reaction. However, under the same reaction conditions reported for the OPSS-PEG (5 equivalents PEG stirring for 3 hours at room temperature in ethanol with the free base form of the LPEI), we observed no product. For that reaction, LPEI in the free base form was obtained by dissolving the hydrochloride salt obtained from Sigma into H2O (100mg in 5mL), then adding 6M NaOH until the final hydroxide concentration of the reaction mixture was 3M NaOH (5mL 6M NaOH added). The resulting precipitate was separated from the supernatant by centrifugation, washed 3 times with 1M NaOH, and then washed several times with deionized H_2O until the wash tested neutral on pH paper. The resulting free base LPEI was dried overnight under vacuum, aliquoted and stored at -20°C for future experiments. In an example of the reaction based on literature precedence, 2mg of base form LPEI (LPEI_B) was dissolve in 100µL of EtOH. 0.5mg PEG-N_3 (5eq) in 100uL EtOH was added. The reaction was stirred for 3 hours at room temperature. The resulting LPEI was then converted back

into its hydrochloride for by adding 400uL 1M HCl. The reaction was then air dried and redissolved in neutral H2O (pH of the solution was 7) before injection onto the FPLC.

Thus, we spent a lot of effort 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 5**). 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. 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 6, next page**).

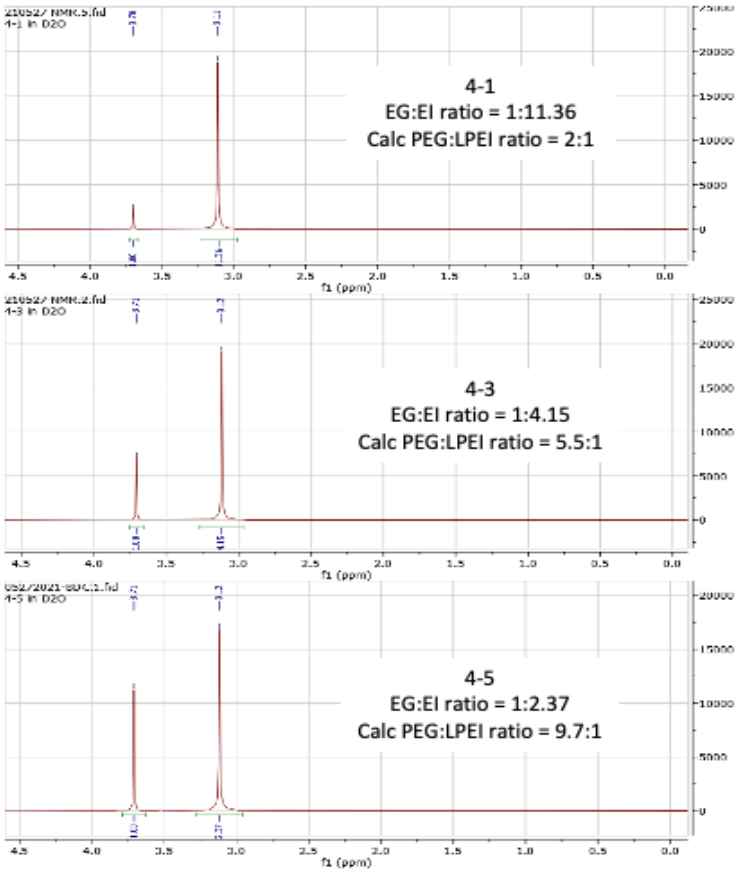

Figure 5: Tuning the EG:EI ratio. These NMRs show differing EG:EI ratios based solely on the amount of PEG added (1, 3, and 5 eqs). All syntheses were done at similar scales (~20mg) and with similar reaction conditions (3 hours at room temp).

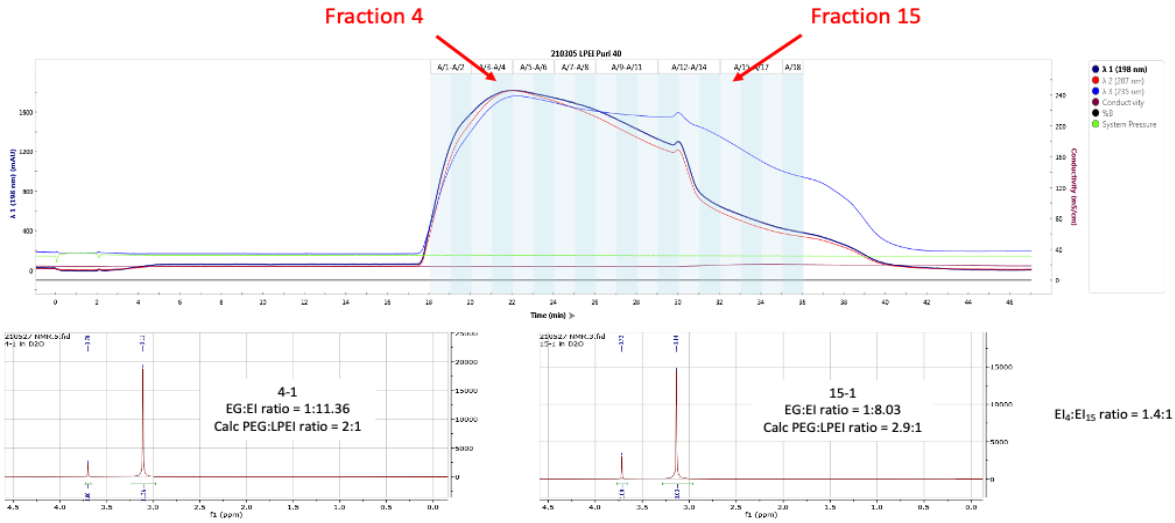

Figure 6: SEC separation of LPEI. The top chromatogram shows the separation of different length LPEI chains via SEC chromatography. The NMRs show differing EG:EI ratios based solely on the length of LPEI chain. Fraction 4 is the longer chain and fraction 15 is the shorter chain. Both syntheses were done 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 produced and validated the novel site specific linker PODS-DBCO (**Figure 7**). Dr. Carney developed the synthesis of the novel PODS-DBCO (**Figure 4**) in anticipation of a successful resolution to the LPEI-PEG conjugation. We have completed the multistep synthesis, purification, and characterization of the PODS molecule. We also spent significant time to optimize the synthesis and to produce an amount of the linker adequate for the remainder of the study.

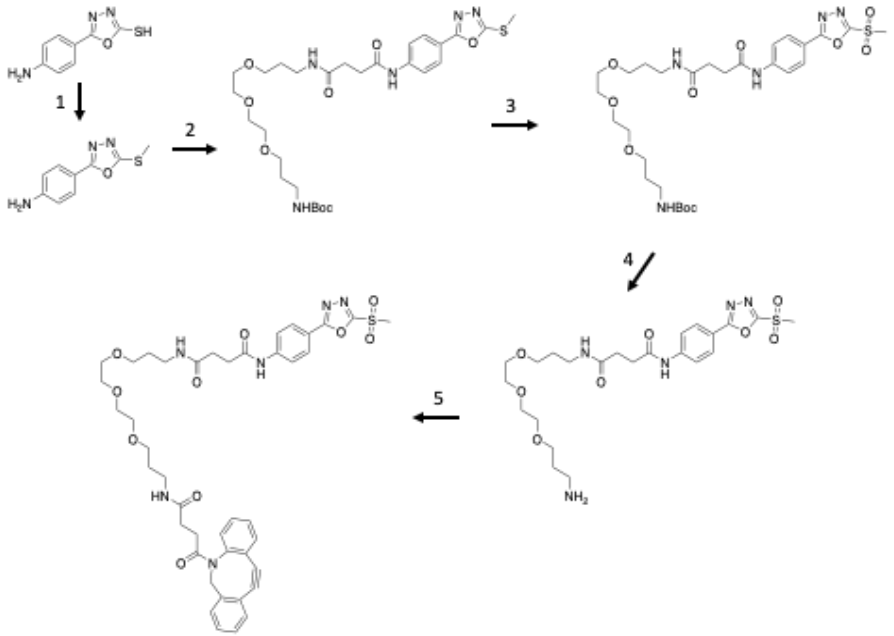

Figure 7: PODS-DBCO Synthetic Scheme. First the thiol group is methylated with iodomethane (1), then the PEG group is added with NHS ester chemistry (2). The sulfur is then oxidized with mCPBA (3) and the primary amine is deprotected (4). Finally, the DBCO is added with NHS ester chemistry to yield the final product (5).

Unfortunately, Dr. Carney left the laboratory in abruptly and unexpectedly due to family concerns in December 2021. After spending two years developing these techniques and expertise, we had to hire and train new lab members to replicate his work and scale up the previously reported conjugates. Ms. Abbriano, a research support specialist in the Houghton Laboratory, worked with Dr. Carney during his final weeks in the lab and thereafter with Dr. Houghton to train in the preparation, purification, and characterization of the LPEI-PEG conjugates. Dr. Kaur, a new postdoctoral fellow in the Houghton Laboratory, was also hired in mid-2022. While she was hired primarily for another project, she too was trained to take over some of the research on this project along with Ms. Abbriano and Ms. Bhatt, who is performing the cell line and related *in vitro* work.

The next steps in the synthesis of our triconjugates was the attachment of the affibody to form the completed triconjugates. Towards that goal, we attempted to screen reaction conditions for the directly conjugating the affibody to the diconjugate under basic conditions. However, this step proved impossible due to low solubility, and we were not able to observe the formation of the triconjugates. For that reason, we decided to first conjugate the PODS-DBCO to the the affibody. This was achieved under mild reducing conditions as reported for other antibody-PODS bioconjugations and confirmed by then reacting a fluoroscein-azide. To test the effects of the conjugation on the binding of the affibody, we performed cell binding assays using the EGFR expressing cell line BxPC3 but we found that the affibody was not demonstrating specific binding. We hypothesized that it may have been due to the long-term storage of the affibody and shipment from our previous institution. We requested that our collaborator send us new aliquots of the affibody to test as it is not commercially available. *However, the laboratory of our collaborator did not have any additional affibody to send us, which led us to consider an alternate path forward to achieve EGFR targeting with our polyplexes.*

As an alternative, we have been pursuing the development of cetuximab and cetuximab fragments that can be directly substituted for the affibody that we had originally proposed to use. To achieve this we have used a commercially available kit (FragIT; Genovis) to generate the f(ab')2 fragments. The kits utilize a proprietary cysteine protease to cleave the antibody below the hinge site yielding a fragment with a conveniently exposed free cysteine to modify with our PODS-DBCO. To date, we have produced the fragments from cetuximab, labeled then with a fluorescent tag, and determined that they do bind to EGFR expressing cells. We have recently optimized the conjugation of the PODS-DBCO to the cetuximab f(ab')2 fragment and characterization of that bioconjugate. We have found that we can tune the number of PODS per cetuximab fragment by simply altering the equivalents of PODS in the reaction which takes place in PBS with a pH of 7.4 over 2 hours. T

Very recently we have worked out the conditions for the next step, which is the formation of the triconjugates by incorporating the PODS-conjugated fragment to the LPEI-PEG-N_3 and then using the fluorescently labeled antagomirs to form the final polyplexes to use for in vitro validation. This is achieved in a "single pot" reaction. The antagomir, LPEI-PEG-N_3, and cetuximab-PODS are mixed in a 0.1:1:2 (antagomir to cetuximab to LPEI-PEG-N_3) ratio in PBS pH 7.4 for two hours. Our spectra from the size exclusion chromatography of the reaction shows formation of the final polyplexes, which is an incredibly exciting results given the wholly new process we had to develop. However, these show that there is likely a mixture of two different products. Based on the size difference, we suspect that there are some PODS-cetuximab with 1 LPEI or 2 LPEI conjugated. We are currently working on conditions for separation of the two species via size exclusion chromatography by reducing the flow rate to more slowly elute and separate the two species. While we expect that a 1:1

LPEI to cetuximab will be best, we will evaluate both via in vitro cell assays to determine which is best for future in vivo experiments.

The size exclusion chromatograms of the full size cetuximab starting material and the fully formed polyplex product are shown in Figure 5. The reduced size (longer retention time) of the polyplex (~125kDa) relative to the full size cetuximab (~155 kDa) is apparent and the small peak arising from the fluorescent antagomir (560nm) aligns with the expected molecular weight of the polyplex. The peak is relatively small due to the small amount of antagomir we used in this pilot study. We are optimizing the conditions to obtain a fully 1:1:1 construct currently. We expect that the separation process will take a few weeks to optimize and that once that is completed, we will be able to rapidly complete Subtask 3 and 5 to wrap up Major Task 1.

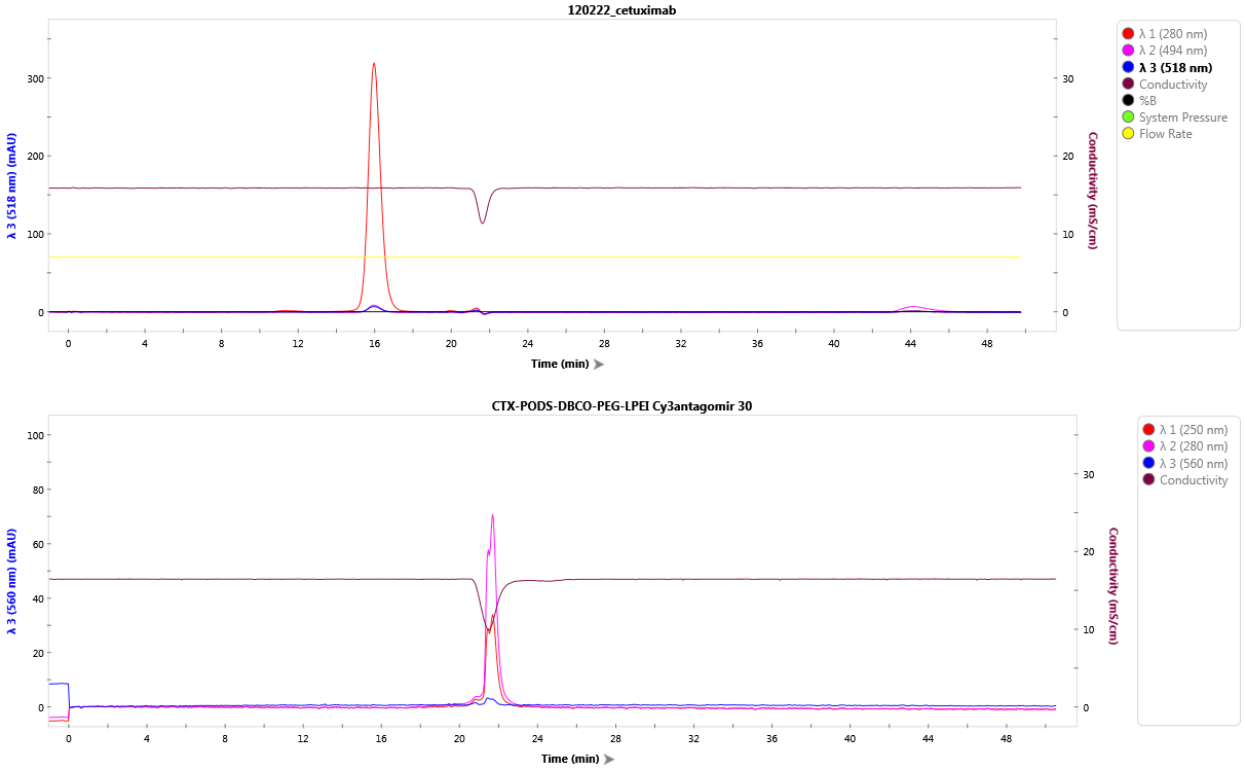

Figure 5. Size exclusion chromatogram of full size cetuximab (top panel) compared to fully formed polyplex (bottom panel).

One caveat to the completion of Subtask 2 is that the supplier who agreed to prepare the antagomirs with DFO for radiolabeling with ^{89}Zr will no longer prepare them citing that it could affect the binding. While we disputed this with them for months, arguing that it is no different than the fluorophore-modified versions, they will not attempt it. We do not have the equipment or expertise

to synthesize antagomirs in our own lab and we will use the fluorophore-modified versions for all our in vitro work as planned.

So that we can use PET imaging to determine the fate of the polyplexes in vivo, we decided to try to use standard conjugation techniques that Dr. Houghton has used for more than a decade to directly label the polyplex and label with ^{89}Zr. Depsite several months of effort to directly conjugate the a bifunctional DFO chelator to the polyplexes, we were never able to achieve a radiolabeling. Unfortunately, this means that *unless the supplier or another contractor is willing to provide the original DFO-antagomir constructs, the originally proposed route for producing the polyplexes for imaging and therapy will not be possible*.

In sum, Subtasks 1-4 were completed as much as was possible but did not yield a polyplex that was suitable for in vivo testing, so this Task remains incomplete.

Major Task 2 (complete)

We also focused substantial efforts on transient transfection of our established cell lines with TOPFlash reporting system. Dr. Houghton and Ms. Bhatt have used a commercially available kit, screening a number of conditions for transfection and detection of luciferase activity in the cell lines. The BPS Bioscience TCF/LEF Reporter kit (Catalog # 60500) was used for transfection of TCF/LEF into the CC-CR cells according to the manufacturer's protocol. The BPS Bioscience Dual Luciferase (Firefly-Renilla) Assay system (Catalog # 60683-1) was used for detection of the Firefly and Renilla luciferase activity in the CC-CR cells transfected with the TCF/LEF reporter or a Non-Inducible luciferase reporter vector according to the manufacturer's protocols. After initial failure of the transfections using the precise instructions from the manufacturer, we also screened additional concentrations of the TCF/LEF luciferase reported vector DNA, amounts of lipofectamine 2000, incubation times, and amounts of recombinant human Wnt3a in order to determine if we could successfully transfect and detect signal in the cell lines. Unfortunately, none of the conditions that were screened led to any indication of successful transfection. As a result, we worked with Dr. Coffey's laboratory at VUMC to develop a stably transfected version of these cell lines to use for the in vitro and eventually the in vivo work outlined in our SOW. The transfection using a lentiviral vector have proven to be a more suitable route and the development of these stably transfected cell lines was recently completed in collaboration with Dr. Coffey. We will soon verify their suitability for these experiments using the same BPS Bioscience Dual Luciferase (Firefly-Renilla) Assay system for detection of the Firefly and Renilla luciferase activity. While this was a slight variation of the proposed work in Major Task 2, it was a major comment by our reviewer in the initial application that turned out to be very pertinent. We believe that these cell lines will be much more suitable for the in vivo work we have planned in the coming months due to their stable transfection rather than transient transfection.

In sum, Major Task 2 was completed in a much more satisfactory, though time consuming, manner and will provide a tool for studying the effects of antagomirs in the future.

Major Task 3 (Partially complete)

Although we have prepared the materials to pursue this aim and planned to begin the experiments in early 2023 once the full polyplex purification had been optimized and we had collected sufficient material for the studies, we have been unable to do so. As planned, the cell lines have been expanded

and enough to place vials in cryostorage, we have developed plating strategies for setting up the asssays, and we have tested the polyplexes. Unfortunately, the modifications were made to the preparation of the polyplexes led to products that were no longer capable of binding to the antigen and thus we were unable to complete Major Task 3.

Major Task 4 (incomplete)

These studies were designed to utilize the stably transfected cell lines that were developed. Our plan was to do a pilot xenografting study to determine the appropriate number of cells to grow suitable xenografts and to use those xenograft models to test the polyplexes that we prepared using our novel methodology. However, the polyplexes failed in vitro validation and were not suitable for their intended use. Thus, it was not possible to complete Major Task 4 as planned.

Major Task 5 (incomplete)

Despite the difficulties and ultimately disappointing results, we plan to publish the results in a single journal article and to present the findings at a scientific meeting in 2024. With the polyplexes and cell line complete, we have enough preliminary data to support future grant applications and we are working to identify appropriate award mechanisms that will allow us to continue this exciting work in the future and to assess its suitability for studying other mRNA mechanisms of resistance.

Significant results to date

- Polyplex synthesis
    - Optimized synthesis conditions for LPEI-PEG-N_3
    - Developed and optimized purification techniques LPEI-PEG-N_3
    - Developed techniques for tuning EG:EI ratios
    - Produced and validated PODS-DBCO
    - PODS-DBCO conjugated to affibody
        - Affibody does not show binding to EGFR-expressing cells
    - Developed a new approach to utilize cetuximab antibody fragments which will provide an easily accessible commercial source of targeting molecules for the polyplexes
        - Did not bind to cells either
    - Completed formation of final polyplexes
        - Optimized purification and scale up
        - Did NOT bind to cellular targets
    - New fluorescent antagomirs delivered

        - DFO-conjugated antagomirs will *not* be available
    - Attempted direct conjugation of DFO to LPEI and fragments for imaging of polyplexes. Conjugation is presumed successful but no radiolabeling was achieved.
- 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 attempted
        - Despite screening conditions, it was found that the cells are not amenable to transient transfection using commercial kits
    - Worked with Dr. Coffey's laboratory to develop a stably transfect TOPFlash CC and CC-CR cell line for use in these studies
        - Clones selected and cultured
        - Scaled up and froze stocks
        - Validated plating conditions for 96-well plate assays
- IACUC and ACURO approval obtained at SBU
- 4 total junior scientists and staff have been trained in all of the requisite skill sets and colorectal cancer in general

    - *FTE levels were actively adjusted (reduced) during training periods to reflect actual work on the project so support would remain available*

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. Originally, Dr. Houghton planned to hire a postdoctoral fellow with experience in these fields, but VUMC refused to allow him to hire the personnel (a second postdoc) guaranteed in his start up contract. As a result, Dr. Carney switched projects at that time and has become an invaluable resource and productive researcher in a very short period of time. The training he received allowed him to carry on the project at Stony Brook University in a seamless manner.

Dr. Houghton has gained didactic training in colorectal cancer biology and imaging by attending the weekly Coffey Group Meeting, weekly GI SPORE meeting, the Epithelial Biology Center monthly seminars, and Vanderbilt Digestive Disease Resource Center (VDDRC) seminar series. 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. Additionally, Dr. Houghton and Dr. Coffey have planned for potential grant applications (e.g. Impact Award) for research that we anticipate we result directly from this project. During the pandemic-related work from home order, we turned our efforts into an exhaustive analysis of the literature and plans for future grants. Dr. Houghton's training under the mentorship of Dr. Coffey provided him an excellent background in colorectal cancer and *Dr. Houghton has recently learned that an NIH R01 for targeted therapy in colorectal cancer will be funded, showing the value that this mentored award had for his career*.

Dr. Kaur and Ms. Abbriano began training in polymer and bioconjugation chemistry as well as didactic training in colorectal cancer biology and imaging with Dr. Houghton. These hands-on trainings were extremely valuable as it prepared them for the work on the project, which required numerous skillsets that they had previously not developed. In addition to learning chemical synthesis techniques, they also learned how to perform numerous purification techniques on poly ionic compounds using ion exchange chromatography, which are new to them. They also expanded their knowledge of colorectal cancer biology. These skills have carried over perfectly to new colorectal cancer projects that have begun in the lab since the expiry of this award.

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 recently (August 2022) become a full affiliate member of the Chemistry Department at SBU where he will be able to recruit students to continue this project long term.

Dr. Houghton has also been 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.

Due to the departure of Dr. Carney shortly after the final project period, I have been training a new laboratory staff member to continue his work while efforts to recruit a new postdoctoral fellow are ongoing. Ms. Abbriano and Dr. Kaur have taken over for Dr. Carney as the primary "hands on" researchers carrying out the development of the polyplexes. They will continue professional development activities suited to trainees.

Based on the Major Tasks in the Career Development SoW, Dr. Houghton has achieved all of the stated goals in terms of didactic, grantsmanship, and personnel management training. Furthermore, Dr. Houghton has developed a strong professional network in the field of colorectal cancer research.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

NOTHING TO REPORT

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

NOTHING TO REPORT – final report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have developed some interesting techniques for the synthesis, purification, and characterization of PEGylated LPEI that can be functionalized. We believe that peptide or small molecule based targeting molecules, rather than biomolecules, will work better because the LPEI likely is inhibiting the biomolecules ability to bind the target.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

NOTHING TO REPORT

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

NOTHING TO REPORT

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

NOTHING TO REPORT

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

There were 4 minor changes to our approach that did not change our objectives or the scope.

1. We have confirmed that the published methodology for synthesizing and purifying intermediates for the polyplexes were not reproducible. We conferred with numerous experts in polymer chemistry, and they confirmed that the published methods were ambiguous. We sought to identify alternative methods, and Dr. Carney developed a novel approach to prepare well-characterized polyplexes, which is reported above.

2. We had to change our approach to the development of the cell lines for testing the polyplexes. The cell lines were not amenable to transient transfection after months of screening conditions and testing commercial kits. Thus, we worked with Dr. Coffey to develop a stably transfected version of the CC and CC-CR cell lines with TOPFlash. This approach was actually suggested by one of the reviewers on our initial application and will provide a more robust model for testing our polyplexes.

3. A third minor change in the approach was necessary due to our loss of access to the affibody. This was previously provided by a collaborator who has since retired, and his laboratory is no longer able to provide material for these studies. We worked to develop antibody fragments that would be amenable to our application. Initial results were promising, showing that we could form the polyplex triconjugates as planned, but they ultimately did not bind to the cells as hoped.

4. A final change has been made necessary due to the companies that originally agreed to prepare and provide functionalized antagomirs. The two companies that provide this service will no longer provide the functionalized versions so we attempted to attach our PET isotopes directly to the triconjugates. This also ultimately failed, likely due to the poly-ionic nature of the components of the polyplex triconjugates.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

The biggest delay has been a result of COVID-19. Staring the first week of March 2020, we were not able to access the laboratory on a regular basis and this persisted through the time we left VUMC on June 3rd, 2020. During that time, I persisted in my career development by attending Dr. Coffey's

virtual lab meetings and other colorectal cancer seminars. We also began preparing for grant submissions that we thought might be possible as a direct result of our proposed research.

Upon arriving at SBU, COVID-19 continued to cause significant delays on a number of fronts. We, and importantly our equipment vendors, were limited in our access to the laboratories for nearly 9 months through Spring 2021. 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. 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. Additionally, the company from which we originally planned to source the antagomirs was acquired and *drastically* increased their prices. We identified a new vendor and the antogamirs were expected to arrive in October 2021, but at the last minute they decided they would not supply the DFO-conjugated antgomirs. At the same time, we found that our cell lines were unsuitable for the transient transfection, despite our best efforts over 6 months in screening conditions with the support from Dr. Coffey's laboratory. For this reason, we collaborated with Dr. Coffey to develop a stably transfected version of these cell lines which was successful but took nearly 1 year in total.

A final delay was caused by the abrupt departure of Dr. Carney from the laboratory. He spent two years developing the methodology for the polyplex synthesis and it took a substantial amount of time to identify and hire new personnel. The new personnel, however, had little to no experience in the field and this required that Dr. Houghton train them to fill the role, which took months of unanticipated time away from direct progress on the project.

In the end, the delays associated with COVID, supplier disruptions, collaborator retirement, and unexpected personnel turnover made it difficult to achieve the aims in a timely manner. However, the goals of career development were met with absolute aplomb and our laboratory has benefitted massively even if the project was not as successful as we hoped.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The restrictions on travel led to decreased expenditures on travel. Currently, we have not charged any travel to the award. Additionally, we have not spent as much as anticipated on radioactive isotopes or consumables. Lastly, we have had personnel turnover in the lab and only recently hired a replacement postdoc so personnel costs were lower than anticipated to date. We do not anticipate any impact on total expenditure. We were granted a NCE to continue the project and have utilized the funds as expected.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

NOTHING TO REPORT

Significant changes in use or care of vertebrate animals

NOTHING TO REPORT

Significant changes in use of biohazards and/or select agents

NOTHING TO REPORT

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

NOTHING TO REPORT

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

NOTHING TO REPORT

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

NOTHING TO REPORT

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

NOTHING TO REPORT

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

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**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

NOTHING TO REPORT

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

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: Jacob Houghton, PhD (PI) – 6 person months per year

Name: Brandon Carney, PhD
Project Role: Research Support Specialist
Nearest person month worked: 12
Contribution to Project: Dr. Carney's developed the synthesis, purification, and characterization of the polyplexes and small molecules.
Funding Support: All work on this project was supported by this award.

Name: Courtney Abbriano
Project Role: Research Support Specialist
Nearest person month worked: 4
Contribution to Project: Ms. Abbriano's laboratory work involved being trained in the areas of polymer synthesis, purification, and characterization. She has also worked with Ms. Bhatt to develop the cell lines.
Funding Support: All work on this project was supported by this award.

Name: Kavita Bhatt
Project Role: Research Support Specialist
Nearest person month worked: 4
Contribution to Project: Ms. Bhatt's laboratory work to date has involved the development of the transiently transfected TOPFlash cell lines and bringing up all cell lines for the project.
Funding Support: All work on this project was supported by this award.

Name: Amritjyot Kaur
Project Role: Postdoctoral Fellow
Nearest person month worked: 3
Contribution to Project: Dr. Kaur's laboratory work has focused on training for the development of the polyplex synthesis, picking up where Dr. Carney left off as well as training in antibody fragment preparation. Dr. Kaur worked on forming the polyplexes.
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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Two pilot awards were awarded to Dr. Houghton since the previous progress report.

Title: **Pb-based Pretargeted Radiotheranotics**

Major Goals: **This is a sponsored research agreement with Viewpoint Molecular targeting with the goal of synthesizing and evaluating novel radioligands for Pb-based pretargeting to support future grant applications in colorectal cancer.**

Status of Support: **ACTIVE**
Project Number: **N/A**

Name of PD/PI: **Jacob Houghton**

Source of Support: **Viewpoint Molecular Targeting**

Primary Place of Performance: **Stony Brook Cancer Center**

Project/Proposal Start and End Date: (MM/YYYY) (if available): **7/2023 – 6/2024**

Total Award Amount (including Indirect Costs):

Person Months (Calendar/Academic/Summer) per budget period = **0.06**

Title: **Development and dosimetry of Pb-203/212 host:guest pretargeting**

Major Goals: **The goals of this project are to evaluate the effects of various chelators on Pb-labeled radioligands' pharmacokinetics and stability as well as to perform dosimetry experiments to investigate the advantages of pretargeting relative to direct labeling.**

Status of Support: **ACTIVE**
Project Number: **N/A**

Name of PD/PI: **Jacob Houghton**

Source of Support: **Office of Vice President of Research (Stony Brook University)**

Primary Place of Performance: **Stony Brook Cancer Center**

Project/Proposal Start and End Date: (MM/YYYY) (if available): **7/2023 – 12/2024**

Total Award Amount (including Indirect Costs):

Person Months (Calendar/Academic/Summer) per budget period = **0.06**

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Organization Name: Vanderbilt University Medical Center (Dr. Coffey)
Location of Organization: Nashville, TN
Partner's contribution to the project: Collaboration with Dr. Coffey to prepare cell lines.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*