Pancreatic Tumor Cell Capture via Dendrimer-Mediated Multivalent Binding and Antibody Cocktail

BY

MEHAR K CHEEMA
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THESIS

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Defense Committee:

David Eddington, Chair
Seungpyo Hong, Advisor, Biopharmaceutical Sciences
Salman Khetani

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURE PAGE

LIST OF ABBREVIATIONS

CTC	Circulating tumor cell
PAMAM	Poly (amidoamine)
EGFR	Epidermal Growth Factor Receptor
EpCAM	Epithelial Cell Adhesion Molecule
CEA	Carcinoembryonic Antigen
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
NHS	N-Hydroxysuccinimide
PEG	Poly (ethyleneglycol)
mPEG	methoxypoly (ethyleneglycol)
PDMS	Polymethyldimethylsiloxane

SUMMARY

In this study, the aim was to combine G7 poly(amidoamine) (PAMAM) dendrimers and a three antibodies targeted specifically towards pancreatic tumor cells, including cell surface proteins Epithelial Cell Adhesion Molecule (EpCAM), Epidermal Growth Factor Receptor (EGFR), and Carcinoembryonic Antigen (CEA). Through integration of the multivalent binding effect via G7 PAMAM dendrimers and the cocktail of the three antibodies, we designed and engineered a CTC capture system that exhibits a high recovery yield compared to existing CTC detection technologies.

Our results demonstrate preliminary findings that the capture surface with G7 dendrimers exhibits a significantly higher recovery yield (60-70%), compared to the surfaces without dendrimers (20-40%). Furthermore, our results also show that the triple antibody system of EpCAM/EGFR/CEA exhibits 15-20% greater recovery yield when combined with G7 dendrimers than the surfaces with each of antibodies immobilized individually.

These findings provide the preliminary groundwork of developing this CTC detection system to be used for blood samples from pancreatic cancer patients, potentially allowing early and accurate diagnosis through enhanced detection and analysis of pancreatic cancer CTCs. Future work of testing this CTC detection system using a variety of clinical cancer patients will validate the clinical impact of this approach.

I. INTRODUCTION

A. Background

Pancreatic cancer has one of the highest mortality rates of any cancers. Current technologies remain inadequate to detect pancreatic cancer early enough for treatment plans to take effect. Only approximately 4% of patients will live past 5 years once diagnosed with pancreatic cancer due to late detection [1]. The main goal of pancreatic cancer diagnostic and clinical treatments is early detection; this is critical due to the associated high mortality rate. Failure to diagnose and late diagnosis both contribute to the high mortality rate, and are some of the many problems new research techniques attempt to tackle and solve.

Progression of pancreatic cancer works through the evolution of precursor lesions and genetic alterations during the process of carcinogenesis. Pancreatic cancer also evolves through papillary mucinous neoplasms and cystic neoplasms [2]. Characterized by high metastatic rates and rapid invasion, pancreatic cancer is near impossible to detect in the early stages. Through tracking the rate of evolution of carcinomas in the pancreas, and further metastasis to other organs, the degree of development can be traced to determine the severity of the pathogenesis and used to determine an accurate treatment plan [2]. The spread of pancreatic tumor cells from the primary site to further areas of the body is facilitated through the bloodstream. Metastatic cells display characteristics of pancreatic carcinoma and display cell surface

markers that reflect these specific tumor cells. Pancreatic carcinomas are characterized specifically by multiple mitogenic growth factors, including epidermal growth factor (EGF) and others including hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) [3]. These surface markers are used as a tool for identifying and diagnosing pancreatic tumor cell occurrence via biopsy techniques. Tracking metastases of pancreatic carcinoma cells to other organs is key for detecting severity of the carcinogenesis and development.

B. CTC use in the clinic

New diagnostic tools use circulating tumor cells (CTCs) to detect the presence of tumor cells traveling from the origin site to other remote organs in the body. As seen in Figure 1, CTCs are derived from the primary site of the tumor and undergo multiple changes and development before reaching the stage known as a CTC, capable of creating metastatic colonies in remote areas of the body after traveling through the blood stream (Fig 1). CTCs have the capability to serve as a biological marker for predicting and monitoring the efficacy of a systemic therapy treatment plan and prognosis in many cases, including breast cancer patients [4,5]. Studies in the past have focused on CTC detection from various pathologies including lung, breast, and pancreatic cancers. These cancers account for many cases of metastatic occurrence in cancer patients and can lead to distant tumor populations making these diseases difficult to treat. Detection of these CTCs and analysis can benefit patients in the early stages of cancer, clinical management of treatment plans, development for personalized

medicine, and also explore the effects and mechanisms of such metastasis [6]. Although CTC count in the blood is low, accurate detection can help develop early diagnostic treatments, since typical detection occurs once the pancreatic cancer has progressed beyond treatment capabilities (Fig 2). Solid biopsies remain the current standard of cancer diagnosis, however, removal of tissue and cells form the body and examination requires experienced surgeons and pathologists, with the invasive nature of the biopsy preventing repeated testing [7]. CTC detection circumvents this obstacle in cancer diagnosis, and speeds up both diagnosis and treatment plan development. Using a simple blood sample for CTC detection compared to repeat solid tissue biopsy allows for increased patient compliance, while reducing risks of complications, such as infection. When used in tandem with current biopsy techniques, CTC detection may serve as a verification method for clinicians to ensure accurate diagnosis.

The presence of CTCs has been previously correlated with distant metastases and an unfavorable prognosis in the cases of biliary and pancreatic cancers [8]. These distant metastatic colonies facilitate the transition of cancer from one organ to spread systemically to the remainder of the body, typically leading to patient death. Once metastatic colonies have established in a variety of organs, typical chemotherapy and radiation treatments cannot effectively target each population and the cancer cells take over the body. Typically, more than 80% of patients with local or distant metastases at time of diagnosis are then not eligible for curative surgical treatment when the disease has progressed too far [1]. Early CTC detection is crucial, even at a low threshold, to ensure treatment plans are effective to lower mortality rates in the future. CTC characterization and enumeration may serve a critical key in diagnosis, tumor

recurrence, detection of chemotherapy resistance, and identification of prognostic outcomes [9]. CTCs have the potential to serve as a marker for pancreatic cancer specifically because they allow for repeat study of tumor genetics and proteomics along with allowing a deeper understanding for the molecular biology of cancer cells [10]. Through combination of CTC detection and tumor biopsy, clinicians will have numerous tools to aid in diagnosis and prognosis of cancer with higher accuracy and rapid results.

C. PAMAM dendrimer use in CTC capture

In the past, nanoscale PAMAM dendrimers have been shown to be an excellent mediator via multivalent binding that is conducted through organizing and orienting ligands, and polymer chain deformation [11]. Multivalent binding is the process of binding multiple ligands to multiple receptors, and plays a crucial role in multiple pathological processes including viral and bacterial pathogens [12]. Due to the effects of multivalent binding, dendrimers facilitate an increased binding affinity that is demonstrated through K_D, dissociation constant, and K_a, association constant. A lower K_D value corresponds to an increased binding strength. These PAMAM dendrimers promote a higher binding affinity by approximately a million-fold via an exponential decrease in the rate of dissociation (K_D), compared to free EpCAM antibody, that has been previously confirmed through SPR analysis [12]. This significant decrease in K_D facilitates a strong binding affinity between G7 PAMAM dendrimers and linked antibodies. One specific mediator of multivalent binding is the partially carboxylated G7 PAMAM dendrimers used in this study, that promote strong multivalent binding that has

been established in previous lung cancer studies [13]. G7 PAMAM dendrimers were chosen specifically for CTC capture based on previous studies from our lab due to their size of 8-10 nm and number of functional surface groups, around 512 in theory to accommodate multiple antibodies per dendrimer to enable multivalent binding [12]. PAMAM dendrimers are characterized by the extensive branching network that is formed through generational addition of amine groups. This extensive branching network forms starting with the core initiator molecule used as the substrate for the initial reaction followed by branching growth. These dendrimers can be tailored specifically through a series of reactions starting at G0, followed by increased branching as the spherical architecture grows.

Previous work from our lab has established that a biomimetic approach may take advantage of the multivalent binding characteristics of the modified generation 7 PAMAM dendrimer and markedly improves the tumor cell capture efficiency [12]. With G7 dendrimer architecture mimicking natural proteins found in the body, the geometry allows for enhanced antibody conjugation. The enhancement in tumor cell capture facilitated by the G7 dendrimers is a direct result of the combined effects of the multivalent binding and efficient protein immobilization, which can result from the spherical architecture of the dendrimers [12]. Through the added binding facilitated by the PAMAM dendrimers, the antibody availability and attachment is promoted (Fig 3). Along with the strong multivalent binding promoted by the dendrimer architecture, this surface capture system is unique in that it uses a three antibody system for effective capture.

D. Existing detection techniques of CTCs

Current CTC detection techniques involve immunological assays against cell surface antigens, molecular assays targeting tumor-derived DNA via PCR or RNA extraction from CTCs and technologies based on the physical and biological properties of cancer cell populations [10]. Immunological assays target cell surface markers, as compared to the antibody capture in this study, and have the ability to target cells expressing cell surface antigens specific to certain pathologies. Tumor derived circulating DNA and RNA are also becoming an effective detection method in determining CTC population and progression. Similar to quantifying CTC number in the blood, detection of circulating tumor DNA and RNA also provide an accurate measurement into presence of tumor cells metastasizing from a specific site of origin to other organs in the body through the blood stream. Presence of these trace DNA markers in the blood allows clinicians to detect presence of CTCs, even while they express a variety of cell surface markers.

Alternative current technologies also include isolation by size, and dynamic capture using microfluidic devices. Isolation by size removes circulating tumor cells using specific size filters tuned for tumor cells. This technology facilitates generalized CTC capture but no cell surface markers are used, and tumor cell determination is based on size alone. Dynamic capture devices use microfluidic channels and dynamic shear flow as the capture environment, while employing markers attached to the channels for CTC capture. Dynamic capture mimics the internal blood stream

environment CTCs use to metastasize to other areas in the body, and allows for real time analysis of how the dynamic flow alters CTC capture. In this study, we developed a technique an assay fine-tuned with a three antibody cocktail against cell surface antigens that present in pancreatic metastatic carcinomas. One unique aspect of this system is the multivalent binding initiated using G7 PAMAM dendrimers to promote further cell attachment.

E. CellSearch comparison

Most current systems employing the CellSearch technology use anti-EpCAM as a CTC capture agent. This method is approved by the FDA and currently is used for prognostic significance in metastatic breast, colorectal and prostate carcinomas [14]. Since the CellSearch system predominantly uses anti-EpCAM, any EpCAM negative tumor cells will slip through and remain undetected. The low number that CellSearch is able to detect may be due to many CTCs having low or no expression of EpCAM [15]. This loss in EpCAM expression is typically due to a transition known as Epithelial-to-Mesenchymal (EMT) transition. Shown in Figure 1, EMT transition is one of the numerous steps that primary tumor cells undergo to reach the stage known as a CTC. During these stages, cells typically expressing epithelial markers such as EpCAM lose this expression and transition into mesenchymal stem cells capable of further migration and invasion throughout the body. There is increasing evidence that EpCAM may be downregulated in clinical samples, and during this EMT transition in metastatic cells, making EpCAM-based detection a biased capture system [16]. Since pancreatic tumor

cells are highly variant, using a multiple-antibody system ensures that a greater number of CTCs can be captured for an accurate diagnostic outcome. By including the additional two antibodies against to pancreatic cancer markers in this experiment, cells expressing low EpCAM levels will still be captured. Further development of CTC detection systems using multiple antibodies for specific cell targeting is the future of this technology.

II. MOTIVATION

The system used in this study for pancreatic metastatic carcinoma employs anti-EpCAM, anti-EGFR, and also anti-CEA, which is pancreatic carcinoma specific. Through the use of the three antibodies and partially carboxylated G7 PAMAM dendrimer binding, this system will exhibit a significantly higher recovery yield of pancreatic circulating tumor cells. Through the development of this assay using a pancreatic cancer cell line, the study provides data for development of this system as a clinical standard that can be used for whole blood samples. When accurately detected, CTCs can be a useful biomarker for a less invasive treatment alternative to the current tissue biopsy samples that are used for diagnosis and treatment, especially in dangerous and unfeasible cases [15]. We hope to develop this system into a model to be used regularly in diagnosis and measuring prognostic outcomes for pancreatic cancer patients.

Through the use of Panc-1 cells as a model for pancreatic metastatic carcinomas, we hypothesize the functionalized cell-capture surfaces with G7 PAMAM dendrimer-antibody conjugates of EpCAM/EGFR/CEA will exhibit the highest recovery yield in comparison to capture surfaces containing singular linked antibodies. By quantifying the presence of G7 PAMAM dendrimer-antibody conjugates on the surface, we will also compare the recovery yield of PEG-antibody conjugates to G7-PEG-antibody functionalized capture surfaces.

Currently, CTC enumeration is not incorporated into the standard clinical practice or management of cancer in any cases [17]. Current standards are biopsy of tissue to to

confirm presence of tumor cells. With the system presented here, once perfected for whole blood samples, can be used in parallel with biopsies to confirm results and prevent repeat, invasive testing. This study serves as the groundwork for the development of clinical CTC capture using whole blood samples from pancreatic patients to accurately detect the degree of metastasis and prognostic outcomes.

III. MATERIALS AND METHODS

A. Surface preparation

Surface preparation followed a previously published protocol from our research group [18]. The functionalized surface was defined using an epoxy glass slide combined with a polydimethylsiloxane (PDMS) gasket, with 6 wells, each holding a volume of 40 microliters. To describe in brief, each surface was functionalized by PEGylation through adding 0.5 ug/mL of heterobifunctional PEG (amine-PEG-caboxylic acid) in DDI water for 4 hours. Further functionalization using G7 poly (amidoamine) was performed via activation with a 1:1 mixture of ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) for 1 hour, followed by partially carboxylated G7 dendrimer addition at a concentration of 50 uM in PBS buffer for 6 hours. G7 dendrimers were used to allow for multiple antibody binding per dendrimer due to their multiple end groups.

Following activation of the carboxylic ends on the immobilized dendrimers through a 1:1 mixture of EDC/NHS, antibodies against human epithelial markers, specifically anti-EpCAM, anti-EGFR, and anti-CEA, were chosen and immobilized at a concentration of 5 ug/mL in PBS overnight on a shaker at room temperature. Antibody conjugation was performed for each antibody singularly, and one condition with all 3 antibodies on one surface. The PEG-dendrimer surfaces were prepared using an epoxy-functionalized glass slide under optimal conditions previously reported from our lab [12,19]. For prevention of nonspecific binding, surfaces were coated with 1 ug/mL

methoxy PEG for another 4 hours following antibody attachment. The fixed volume used in all experiments was 40 uL per well. All incubation was carried out at room temperature under constant, slow shaking. Washing performed between steps was performed with DDI water and PBS to remove any residual chemicals. If the surface wasn't used immediately, all surfaces were stored in 4 degrees C in PBS until use.

B. **Design**

Experimental design was based on previous CTC capture devices developed in our laboratory [12]. An epoxy glass slide was chosen as the surface substrate, followed by addition of the PDMS gasket to outline the wells for the solution volume of 40 microliters. The PEG conjugation and EDC/NHS opening of carboxyl groups for the G7-PAMAM dendrimers to attach to, and the dendrimers were also treated with EDC/NHS to open up the carboxyl groups for antibody attachment at various points to the dendrimer surfaces (Fig 4 and 5). Once attached, the fully functionalized surfaces were ready for cell attachment and incubation. Each slide had 6 outlined wells, maintained for static flow conditions. No dynamic conditions were used in these experiments.

C. **Cell Culture and Binding Assays**

Panc-1 cells were grown in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator at 37 degrees C and 5% CO2. Panc-1 cells were

used as the pancreatic tumor cell model. To fluorescently label the Panc-1 cells for experimental use, the cells were labelled with Calcein AM for 30 minutes prior to the experiment. The final cell concentrations used per well were 50 cells/well, 200 cells/well, and 1000 cells/well.

For the binding assay, each antibody immobilized surface was incubated with the mentioned above cell concentrations for 2 hours in an incubator at 37 degrees C. Cells that remained unbound were removed during a gentle PBS wash following incubation. Determined by the loading cell number, once cells were imaged and counted, recovery yield in % was calculated based on the number plated.

D. **Characterization**

G7 dendrimer characterization was performed using ^{1}H-NMR in D2O using a 200 MHz Bruker DPX-400 spectrometer as shown in Figure 3. Surface characterization of functionalized antibody-dendrimer conjugate surfaces was previously performed, and the same protocol was followed for surface preparation in this study [12]. SPR analysis was conducted for dendrimer-EpCAM conjugates in the previous work to quantify antibody-dendrimer conjugation.

E. **Microscopy**

All microscopy was conducted on a fluorescence microscope using Calcein AM as the tagging fluorescent agent for the cells, and light excitation at a wavelength of 495 nm and a 516 nm emission spectra.

IV. RESULTS

Our results began with characterization of the dendrimers using ^{1}H-NMR to analyze the chemical composition of the dendrimer groups. Carboxylation was required to open up carboxyl groups at the ends and to use remaining amine ends for antibody conjugation, for all 3 antibodies used in this experiment, using a protocol previously outlined for the G7 PAMAM dendrimers [18]. Peak shifts are seen for both the surface amine groups and carboxyl groups used to facilitate antibody binding (Fig 6). The partial carboxylation increased antibody binding to the dendrimers, while also increasing antibody availability to the pancreatic cancer cells. This partial carboxylation takes advantage of the spherical dendrimer architecture, and the G7 dendrimers extensive branching allows for optimal antibody binding to the surface of the dendrimers to increase cell capture

To effectively analyze the results for circulating pancreatic tumor cell capture modeled *in vitro* with Panc-1 cells, the primary determination needed was to ensure no non-specific binding was occurring. To avoid this affecting the results, an Immunoglobulin-G (IgG) assay was used, linked directly to the PEG-G7 dendrimer conjugates to target any non-specific binding that occurred with the Panc-1 cells. Using a prolonged mPEG treatment of 6 hours was a slight modification made for the IgG assay, and it proved to show minimal to no binding when 200 cells/well were plated, as seen in Figure 7. Cells were visually confirmed before the PBS wash via fluorescence microscopy, and once the wash was performed, all unbound cells were washed away.

To further establish the purpose of G7 PAMAM dendrimers, CTC recovery yield was examined using antibodies linked to PEG only and then antibodies linked to dendrimers. The multivalent binding effect that facilitates the higher quantity of antibody binding was demonstrated by the recovery yield of G7 linked capture (60-70%) compared to PEG-only linked antibodies (20-40%), as seen in Figure 5. The capture data also demonstrated the purpose of a combined antibody system of CEA/EGFR/EpCAM. Recovery yield data was collected from 3 cell concentrations: 50, 200, and 1000 cells/well. In all 3 cell concentrations, the same data trend is displayed (Fig. 8).

For the comparative analysis of each antibody alone to the antibody cocktail, each antibody was used separately linked to PEG-G7 PAMAM denrimer prepared capture surfaces, for 3 cell concentrations of 50, 200, and 1000 cells/well. As seen in Figure 6, aEpCAM, aEGFR, and aCEA displayed low recovery yield values (50-65%), while the antibody cocktail of aEpCAM/aEGFR/aCEA displayed a much greater recovery yield (75-100%). These results demonstrate greater recovery yield when using the three antibody cocktail, along with the G7 dendrimers to promote multivalent binding of the antibodies to the pancreatic tumor cells (Fig 9). Through these series of experiments, it is clear singular functionalized surfaces may not be as effective at capturing CTCs since there are a variety of cell surface markers that may be targeted to promote capture compared to using one cell surface marker.

V. DISCUSSION

Invasive pancreatic carcinomas that lead to metastases are the cause of mortality due to pancreatic cancer. Late detection is one of the key flaws in pancreatic cancer diagnosis and treatment. As technology progresses and more effective cancer treatments are developed, there is a higher incentive for early detection of these fatal diseases. Through detection of pancreatic CTCs, the mortality rate of this disease can be significantly decreased through rapid detection and treatment. To replace current standards of biopsies that require skilled surgeons and practitioners, the CTC collection and analysis would only require a simple blood sample collection, and a laboratory technician to run the blood sample through this platform using a multi-antibody system designed specifically for pancreatic cancer.

A. Dendrimer Application and Purpose

The protocol discussed in this study builds upon previous work conducted using dendrimer technology to promote CTC binding. In the past, dendrimers have been used in combination primarily with singular antibodies including aEpCAM and aEGFR for CTC capture [12,13,19] and the beneficial value of employing the use of dendrimers has been proven through multiple studies and applications. In choosing the three antibodies used in this study, aEGFR and aEpCAM have been previously studied in this lab in combination with G7 dendrimers to promote CTC capture and have proven successful

[12]. Carcinoembryonic antigen was chosen as a novel capture reagent specifically tailored towards pancreatic cancer cells to enhance recovery yield.

This preliminary study establishes the beneficial value of combining such dendrimer technology along with an antibody cocktail tailored specifically to pancreatic cancer. The spherical dendrimer architecture functionalized with antibodies on the surface through EDC/NHS carboxyl group opening allows for greater antibody attachment to the surface. To verify we had functionalized the dendrimers and antibodies to the surface together, functionalization and binding avidity of these dendrimers was previously confirmed through G7-PAMAM-aEpCAM conjugates via SPR analysis, and the same protocols were used to prepare the surfaces used in this experiment, with the inclusion of new antibodies [12].

Through these established protocols, this experiment took advantage of the multivalent binding that is facilitated via the G7 PAMAM dendrimers, along with the combination of typical epithelial tumor cell markers including EpCAM, EGFR, and CEA. With the inclusion of novel antibodies in this study, we proved that multiple antibodies work more effectively in combination with both each other and the PAMAM dendrimers. The addition of the dendrimers to PEGylated surfaces was proven in this study, and previous work from our lab, to promote higher antibody immobilization than just PEG alone, even under identical protein immobilization conditions, proving an additional benefit of employing the use of G7 dendrimers [12]. This study built upon previous groundwork established using dendrimer technology as an effective CTC capture tool, and established the benefits of this technology further by the addition of three antibodies

targeting pancreatic tumor cells. These results serve as preliminary groundwork for pancreatic CTC targeting.

B. Innovation from previous technology

The novelty in this system lies in the specificity towards pancreatic cancer and combination of multivalent dendrimer binding along with a tailored three antibody cocktail to promote effective circulating tumor cell capture. Through testing three variant cell concentrations, it is clear that even at low cell counts, a common characteristic associated with CTC populations, this system is still capable of capturing above 80% of plated pancreatic tumor cells. Most circulating tumor populations in the blood maintain low cell counts and are difficult to detect using typical singular antibody detection.

While a static system that we used as the experimental set-up does have potential drawbacks, the advantages shown here are high recovery yield and ability to visualize the attached cells with ease. Unique to our system is also the addition of these three antibodies linked to G7 PAMAM dendrimers for increased antibody availability. The spherical architecture facilities a greater amount of antibody binding, and leads to greater recovery yield of cancer cells. Our results can be used to confirm the only cells recorded were targeted Panc-1 cells via specific cell markers and PBS washing following capture. One cell line was maintained as the testing population for pancreatic tumor cells, and while this system only models an *in vitro* cell culture model of pancreatic cancer, the next step is to develop a model for blood samples from

pancreatic cancer patients and determine the efficacy of the developed system presented here to clinical cases.

C. CTC Applications in alternate studies

Many diagnostic methods are moving towards CTC detection assays as a method to verify results of the current clinical practice of patient biopsies. While patient biopsies are the clinical standard, CTC detection is less invasive and requires simple laboratory techniques to analyze results. Employing both techniques can be used to confirm results and ensure rapid, accurate diagnosis. Typically, such CTC detection involves the use of CellSearch, the standard capture assay that uses aEpCAM as a capture agent. Recently, it has been discovered this should not remain a gold standard, due to the higher effectiveness of combination antibody systems.

A recent study employed the use of Mucin-1 in combination with EpCAM as a capture system in parallel for pancreatic CTCs [16]. In combination with our study, both provide experimental evidence of the advantages seen in using multiple antibodies as a capture reagent versus singular antibody capture. Whether using the multiple antibodies in parallel or functionalized together onto one surface, the advantages to multiple antibody targeting of specific tumor cell populations has shown advantages when targeting certain cancer pathologies. Multiple antibody use in recent work has been used to capture a variety of tumor cells, by targeting the heterogeneity of such cells and the cell surface proteins they are expressing [20]. Combining the multiple antibody cocktail with the novel PAMAM partially carboxylated dendrimers to promote cell

binding, the system presented in our work has shown the benefits and advantages over current technologies.

D. Comparison to ISET

Alternative approaches to CTC capture have used isolation by size technology that allows for compensation of cells that are not expressing EpCAM as well, by isolating based on a predetermined size constraint of typical CTCs and microemboli [15]. Isolation by size is also capable of detecting higher CTC numbers in patients when compared to CellSearch capture number. ISET technology is marker independent, so the advantage of our system in comparison is specificity to pancreatic tumor cells circulating in the blood. With ISET technology, differentiation or specificity of pancreatic CTCs is near impossible, with any cell within the size dimension getting through the filter. In most clinical cases, origin of the cancer and early detection is critical. When using ISET technology, detection may be delayed until the cancer has metastasized to alternate areas of the body and there could be multiple circulating tumor cell types in the blood. ISET capture has exhibited recovery yield of CTC populations comparable to the percentages seen in this study, but with the generalized approach of capture by size with no specific cell markers, it cannot be used to verify where the tumor cell population originated from. The unique combination of EpCAM/CEA/EGFR allows the system presented in this study to target pancreatic origin tumor cells, and can be applied to aid clinicians to detect tumor cell populations in the blood with greater ease and rapid detection. Pancreatic cancer requires early detection, making capture of pancreatic

CTCs with the appropriate markers key, and our system has indicated those results with a high recovery yield.

E. Dynamic vs. Static capture

The system examined in this study remains a static capture system and presents slight disadvantages when compared to a dynamic capture system. Systems similar to dynamic flow in the body provide a higher degree of accuracy when modeling the circulation of these tumor cells in the body in real time. One such system has shown that pancreatic tumor cell capture can be enhanced using a microfluidic mixing chip that employs the use of the standard aEpCAM along with using mixing to capture circulating tumor cells [21]. However, one drawback of this system in comparison to the one used in this study is that at any flow rate higher than 1.5 microliters per second, the capture efficiency drops dramatically below 80% so even with a higher likeness to the microenvironment these tumor cells are circulating in the body, static flow remains an effective method when measuring presence of CTCs for pancreatic cancer patients.

The capture method used in our study involves static capture along with multiple antibodies versus aEpCAM alone, allowing the synergy of such antibodies along with the dendrimer binding to provide advantages in this system over dynamic capture devices employing singular antibodies. Future work using our experimental set-up could develop a dynamic flow chamber to test the functionalized surfaces and how efficiently the dendrimer-antibody conjugates capture pancreatic CTCs under dynamic conditions to mimic natural blood flow through the body which facilitates metastases.

F. Antibody selection for CTCs

There is mounting evidence that CTC populations are heterogeneous and co-expressive markers and CTC detection can be useful for disease prediction, monitoring, and response to many treatment therapies [22]. The targeting of heterogeneous cell populations derived from pancreatic carcinoma cases will allow clinicians to make more accurate diagnoses and disease prediction, along with concrete determination of the effectiveness of any treatment on the CTC population. The system presented here allows clinicians to use a non-invasive, antibody dependent system that is facilitated via dendrimers that promote further binding capacity along with three targeted antibody targets for specific pancreatic CTC capture. When selecting the three antibodies that were used in this experiment, EpCAM and EGFR were chosen based on high expression in CTCs of all types. Previous studies have shown epithelial cell adhesion molecule expression may vary between different types of carcinomas, with cases of both good or poor prognostic outcomes [15].

In many studies, the use of EpCAM is the standard in detection of CTCs, but in addition, adding the CEA allowed us to further develop the system to tailor it specifically to pancreatic tumor cells that are metastasizing to other areas of the body through the blood stream. Metastases formation is conditioned by cancer cells overcoming various stressors and surviving and flourish in in new and hostile environments [23]. Through the changes cells undergo to survive these harsh conditions, specificity in cell targeting allows determination of tumor cell origin. Demonstrating the key effects of multiple

antibodies in conjunction with the multivalent dendrimer binding was the goal of this study, and we have shown that there is a high recovery yield in comparison to singular antibodies when using dendrimers. We have also demonstrated the advantages of using a multiple antibody cocktail in comparison to systems that only employ singular antibody capture or size based CTC capture.

G. Additional testing

One drawback of this system that we have developed is the *in vitro* work is the only data collected so far. Analysis of CTCs from clinical blood samples is critical in analyzing the effectiveness of a capture system. Previous work has outlined CTC detection through blood samples, using mixing chips, ISET technology, and alternative microchip technologies that are capable of analyzing whole blood samples [15,21,24]. The effectiveness of these technologies has proven the advantages of CTC detection over invasive biopsy techniques. Whole blood analysis parallels the natural CTC environment in the body, rather than detection through cell lines developed specifically for pancreatic carcinoma analysis. Our system has only been applied to Panc-1 cell lines so far, and to accurately make a determination on the effectiveness in the clinic, whole blood samples would be used for analysis in the upcoming stages.

VI. CONCLUSIONS AND FUTURE WORK

Through this study, we have shown preliminary results that a pancreatic CTC capture device using multivalent binding facilitated by G7 PAMAM dendrimers linked with a multiple antibody system that binds both aEGFR, aEpCAM, and aCEA exhibits an increased recovery yield of pancreatic cancer cells compared to singular antibody capture and PEG-antibody based capture. Our findings contribute toward the development of a clinical device for CTC capture to be used for whole blood samples that have also been explored using other antibodies through our previous work [12, 19]. The previous tested CTC devices have been explored with clinical whole blood samples, so future work from this study will develop this CTC capture device for whole blood analysis from pancreatic cancer patients.

One drawback of the current system demonstrated here is that cells that are leaving the pancreas and metastasizing to other organs undergo a transition called Epithelial-to-Mesenchymal transition (EMT). These EMT transition cells have been shown to slip through CTC detection methods in the past [25]. Since the cells are transitioning and cell surface markers are changing as these cells progress through the metastatic process, EMT transitions must be taken into account when using CTC detection as a method of prognosis and cancer progression.

Once this system is tested using whole blood samples to capture pancreatic CTCs, alternative markers targeting EMT cells that express variant cell surface markers can be studied. Typically, there isn't one specific tumor marker that consistently and specifically is expressed in all primary tumors of a specific malignancy, CTC capture

systems must take into account the varied marker expression and find a way to target all necessary markers to accurately identify CTC origin [26]. Larger sample sizes including the whole blood samples are further improvements upon the work presented here that can be addressed in future studies. The potential for these CTC detection devices to be applied as the new standard for metastatic diagnosis and prognosis is vast and can benefit both the clinicians and patients and reducing multiple invasive biopsies that cause patient discomfort and hassle for clinicians. Once this system has been further developed and tested with whole blood samples, application in the clinic can become standard practice as these CTC devices are tailored specifically for certain cancers and considering cell transitions through metastasis.

FIGURES

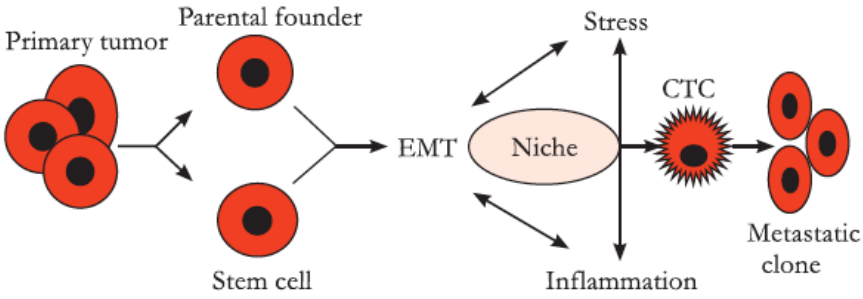

Figure 1: CTC generation from primary site of the tumor, through a progression from the site of origin through EMT transition into CTC and metastatic colonies migrating to far regions of the body [23]

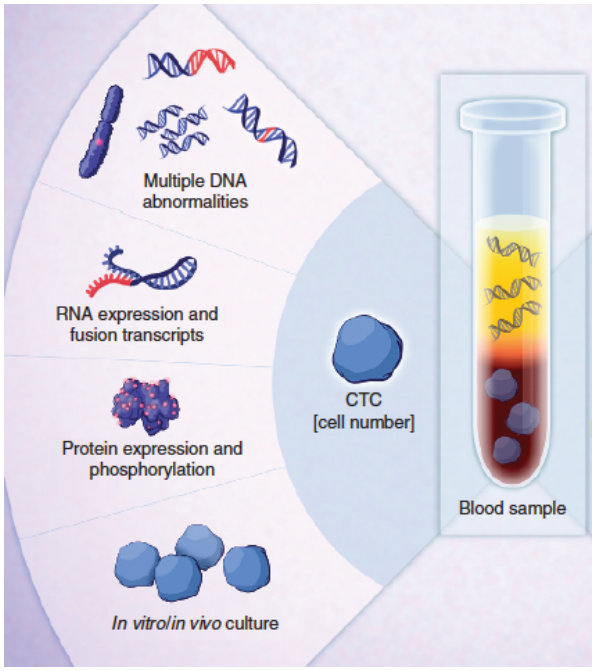

Figure 2: Schematic of CTC application in cancer diagnosis; isolation of CTC can allow for molecular analyses to apply for variant treatment strategies [11]

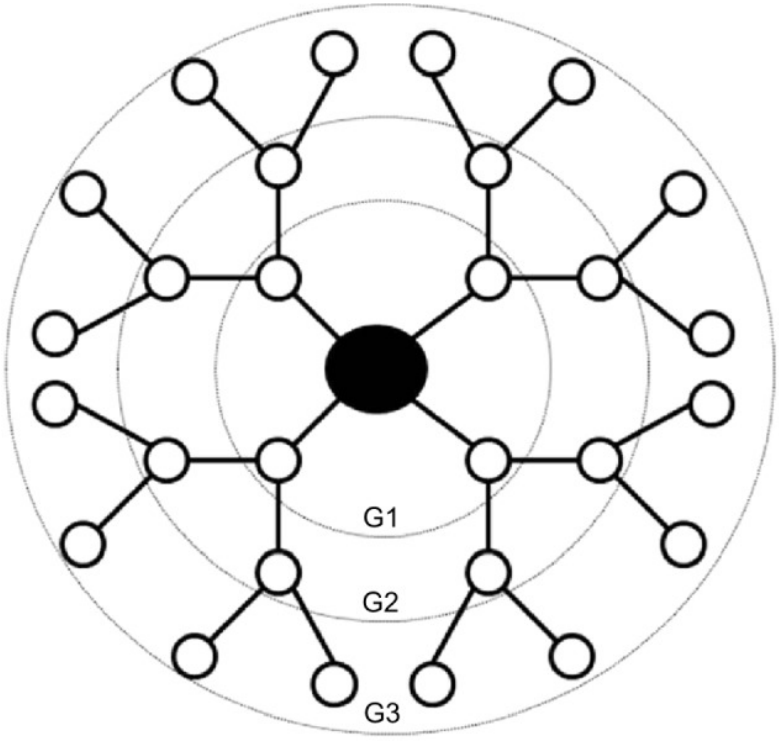

Figure 3: Dendrimer schematic showing generational changes G1-G3 [27]

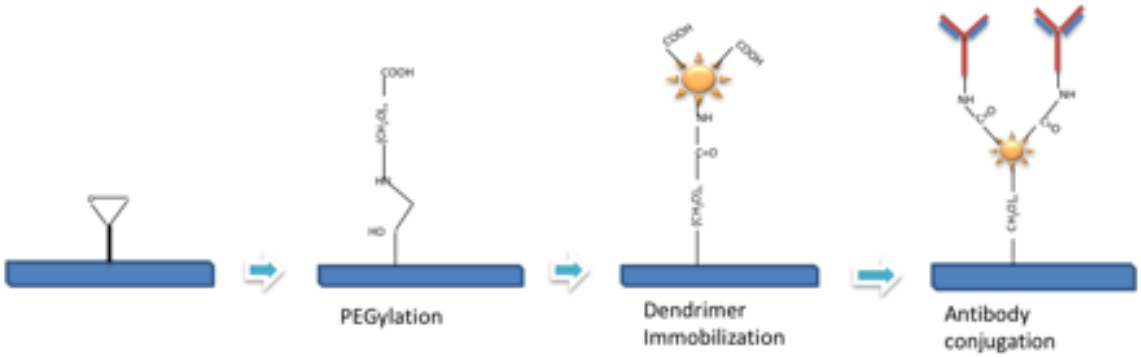

Figure 4: Experimental synthesis surface preparation using PEG linked to PAMAM dendrimers and an antibody linked with open carboxyl groups treated by EDC:NHS

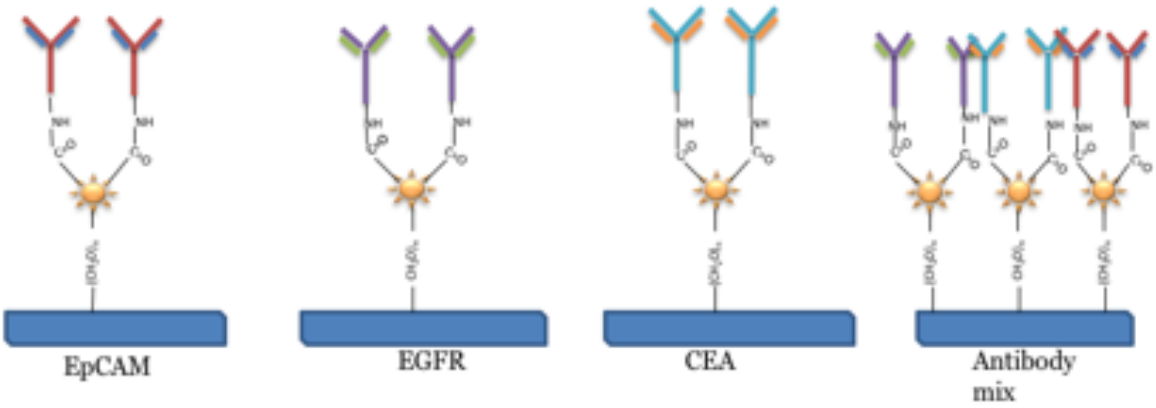

Figure 5: Surface schematic detailing the use of each antibody: Epithelial cell adhesion molecule (aEpCAM), Carcinoembryonic antigen (aCEA), and Epidermal Growth Factor Receptor (aEGFR)

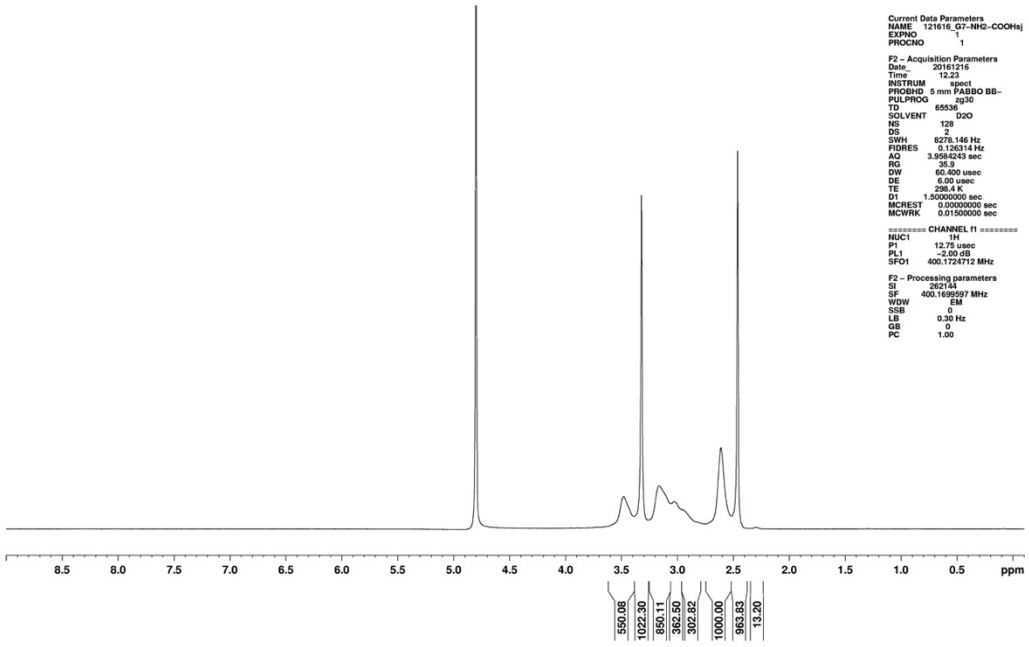

Figure 6: ^{1}H-NMR analysis of G7 partially carboxylated poly(amidoamine) dendrimers

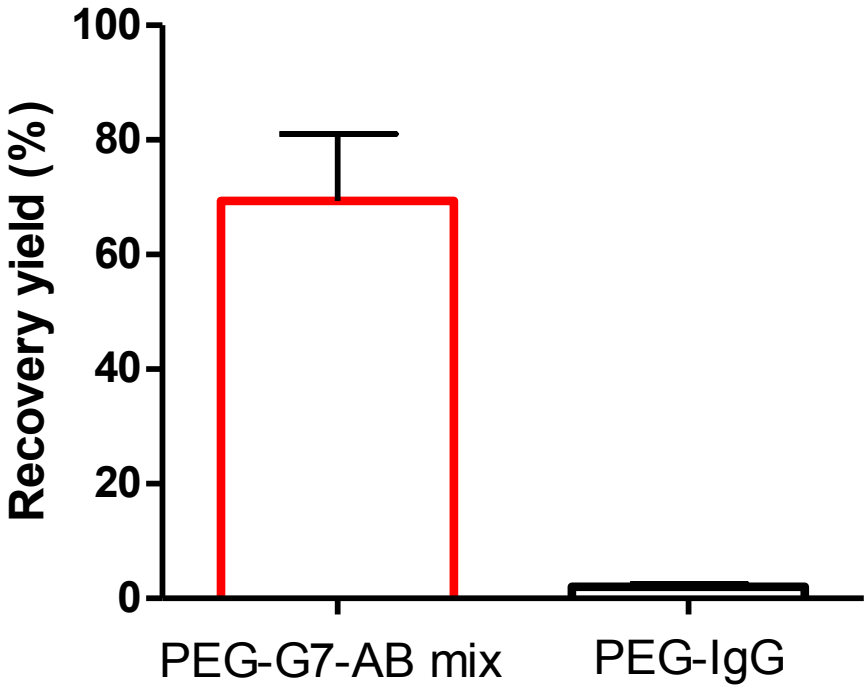

Figure 7: IgG non-specific binding comparison for 200 cells/well, n=6 St.Dev= 1.1143

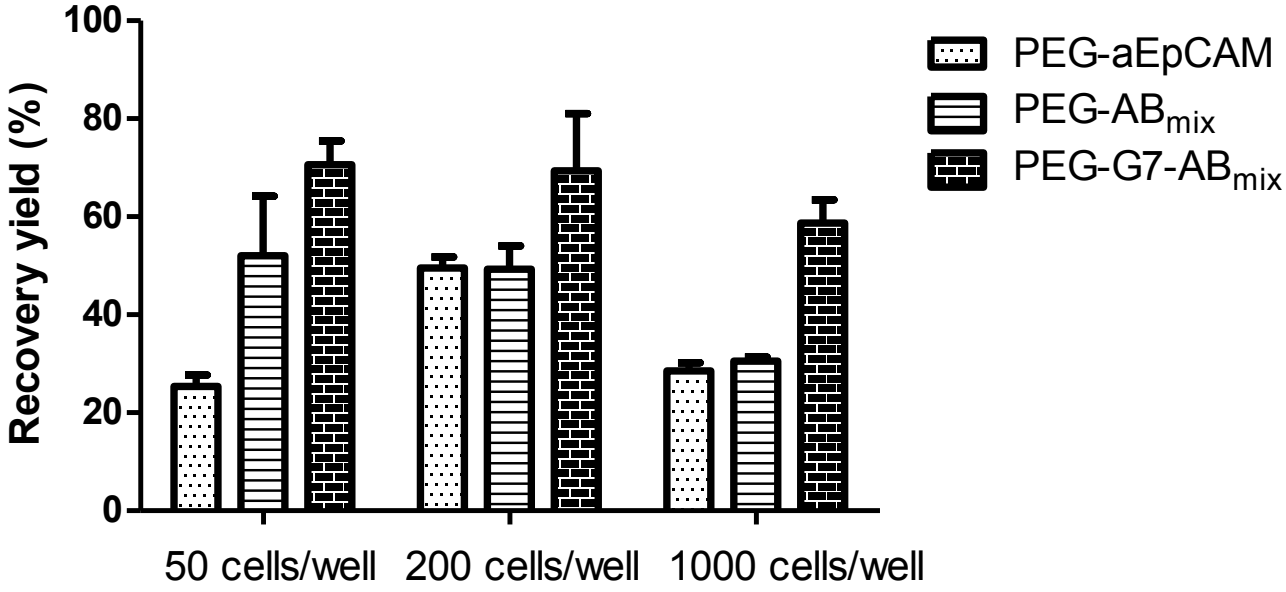

Figure 8: Comparison of PEG-EpCAM and PEG-Antibody mix based capture vs PEG-G7-Antibody mix binding; 3 cell concentrations- 50,200,1000 cells/well

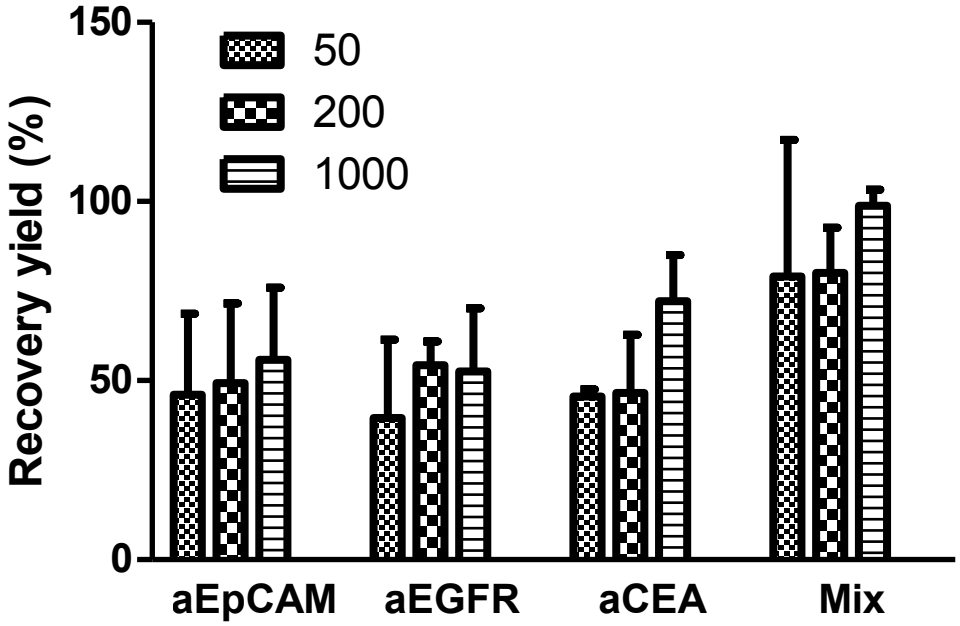

Figure 9: Recovery yield using functionalized surface at cell concentrations of 50, 200, and 1000 cells per well, functionalized with G7-aEpCAM, G7-aCEA, G7-aEGFR, and Antibody mix

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VITA

NAME:	Mehar Kaur Cheema
EDUCATION:	B.E., Biomedical Engineering, Stony Brook University, Stony Brook, New York, 2015
	M.S., Bioengineering, University of Illinois at Chicago, Chicago, Illinois, 2017
WORK EXPERIENCE:	Research Assistant, University of Rochester Medical Center, Rochester, New York June-August, 2013-2015
TEACHING EXPERIENCE:	Department of Biological Sciences, University of Illinois at Chicago; Microbiology Laboratory for Undergraduates, 2016-2017
	Department of Biological Sciences, University of Illinois at Chicago; Cell Biology Lecture, 2016
HONORS:	Alpha Eta Mu Beta Honor Society Member Chicago, Illinois 2016
PROFESSIONAL MEMBERSHIP:	American Association for the Advancement of Science,
PUBLICATIONS:	Burke, Kathleen A., et al. "Second-harmonic generation scattering directionality predicts tumor cell motility in collagen gels." *Journal of biomedical optics* 20.5 (2015): 051024-051024.