OPTIMIZATION OF ACTIVITY ASSAYS FOR SPECIFIC ACTIVITY OF EGFR INCORPORATED INTO NANODISCS

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I. Abstract

Membrane proteins are an essential part of many biological processes that are areas of interest for research. Unfortunately, membrane proteins are difficult to study because once removed from the native cell lipid bilayer, the proteins tend to misfold and aggregate, leading to loss of function. Nanodiscs are soluble, scaffold protein-stabilized lipid bilayers that provide advantages in the study of membrane protein structure and activity.

Epidermal Growth Factor Receptor (EGFR) is a type of receptor tyrosine kinase (RTK) membrane protein. When RTKs mutate, they can lose dependence on extracellular signals and become constitutively activated, which allows them to become contributing factors in many cancers, including non-small cell lung cancer. There is interest in subjecting RTKs to inhibition by tyrosine kinase inhibitors (TKIs) as a potential cancer treatment.

My overall goal is to create library Nanodiscs using cancer cells from patients, which would, in theory, have all of the membrane proteins from the initial cell incorporated into Nanodiscs, and measure the ability of different TKI therapies to decrease EGFR activity. This would allow treatment trial and error to happen *in vitro* rather than *in vivo*, minimizing the trial time and ill effects for patients. However, before we could apply this system to cancer patient samples we need to optimize the activity assay protocols, through a series of diagnostic activity assays to determine the best conditions for measuring high EGFR activity with minimal background interference.

II. Introduction

A. Nanodiscs

Membrane proteins play an essential role in many biological processes, including cell-cell communication, energy production and signal transduction. Specific membrane proteins can be of particular interest in a study, due to suspicion in contributing to a disease or being a target for pharmaceuticals.[1] However, membrane proteins are notoriously difficult to study because once removed from the native cell lipid bilayer, the proteins tend to misfold and aggregate, leading to loss of structure and function. Nanodiscs are soluble, scaffold protein-stabilized lipid bilayers that demonstrate many advantages for studying membrane protein structure and activity. These model membranes consist of lipids and a belt-like membrane scaffold protein (MSP) to study structurally stable and active membrane proteins. Nanodiscs allow access to both sides of the lipid bilayer and the bilayer lipid composition can be precisely controlled, along with the stoichiometry and size. Nanodiscs have become a preferred lipid bilayer mimic system for studying membrane proteins for these reasons.[2]

Nanodiscs are created by slowly removing a solubilizing detergent from a mixture of phospholipids, membrane proteins, and membrane scaffold protein (MSP). This detergent removal process takes 2 to 18 hours, depending on the detergent identity, lipid content and the protein of interest[3]. Nanodiscs will self-assemble with a membrane protein integrating into the phospholipid bilayer wrapped in amphipathic MSP upon removal of this detergent.[4] Many classes of membrane proteins have been successfully integrated in Nanodiscs thus far, including partial-length EGFR, which has been shown to be stable.[5,6,7]

Most previous Nanodisc research has required an isolated and purified protein of interest, usually using a recombinant protein expression system.[5,8] This long and tedious expression step

can be avoided by using whole cell lysate to incorporate membrane proteins into Nanodiscs directly from the cell membrane, allowing membrane proteins to remain in a more native environment throughout the formation of Nanodiscs.[9,10,11] This has many advantages over the recombinant systems. Since certain proteins require a specific lipid composition to remain functional, the ability of native lipids to remain associated with these membrane proteins could help prevent the loss of structure and function. Membrane proteins isolated directly from membranes are also full length and have all post-translational modifications, both of which are common issues when trying to recombinantly express membrane proteins.[8]

Using whole cell lysate creates what has been called Library Nanodiscs, as theoretically all of the membrane proteins from the initial cell can be incorporated into Nanodiscs. These library nandiscs are used for both studies of all membrane proteins in a cell and when the purification of a specific membrane protein of interest may be difficult to obtain using recombinant expression.

B. Epidermal Growth Factor Receptor and Non-Small Cell Lung Cancer

EGFR is a type of receptor tyrosine kinase (RTK), a class of integral membrane protein that deliver extracellular signals into the intercellular environment through conformational changes and phosphorylation that triggers a signal pathway, which ultimately leads to cell proliferation and other processes.[12] When certain RTKs mutate, they can become no longer dependent on the extracellular signals and can be constitutively activated. This mutation or native protein overexpression turns these kinases into potent oncogenic factors that are contributing factors in many cancers. There is significant interest in subjecting RTKs to

small-molecule inhibition by tyrosine kinase inhibitors (TKIs) to potentially prevent these downstream cascades in various types of cancer.[13]

About 15 percent of non-small cell lung cancer (NSCLC) patients (314,000/year) have EGFR mutations believed to be part of the driving force in cancer growth. There has been significant research into treating NSCLC patients with a variety of TKIs, which often are initially successful but eventually stop working. This change in efficacy is caused by further EGFR mutations which cause the TKIs to no longer be able to interact with the protein to prevent signal transduction. Inhibition of EGFR kinase activity with first- and second- generation TKIs tends to be effective for patients expressing either L858R (a leucine to arginine mutation at amino acid 858) or an exon 19 deletion, the two most common initial EGFR mutations in NSCLC. After a few months to a year of treatment, most patients develop resistance to the treatment. This is often due to a secondary mutation in EGFR at amino acid 790 from a threonine to a methionine (T790M). These patients will initially respond to osimertinib, a third-generation TKI that binds covalently to the EGFR, but eventually, patients develop resistance to this treatment as well, caused by mutation C797S, which is the amino acid where osimertinib binds.[14]

In theory, we should be able to create library Nanodiscs using the cancer cells of patients and measure the ability of different therapies to decrease EGFR activity. This would allow trial and error to happen *in vitro* rather than *in vivo*, minimizing the trial time and potential ill effects for the patients. However, before we could apply this system to cancer patient samples we need to optimize the Nanodisc formation and activity assay protocols for less-complicated and more well-controlled systems. By doing this, our research would contribute to individualized oncology, which aims to get the patient their best therapeutic treatment based on their unique genomic and molecular information. In this case, we would be using protein-based

measurements to provide clinically-actionable information in order to direct the most effective course of treatment. This could be exceptionally helpful with NSCLC since treatments often become ineffective since the cancer often develops resistance, especially within the EGFR protein.

For our work, the Nyati lab in the Radiation Oncology Department at the University of Michigan has provided us with Ba/F3 cell lines, a type of mouse leukemia cell line that does not natively express EGFR, with each of the EGFR mutations of interest expressed.[15] The current focus is the optimization of activity assays to measure the most activity possible from the EGFR incorporated in the Nanodiscs, without a significant amount of background noise. The ability to determine the activity of just the membrane protein of interest from a patient and how this protein responds to specific drugs can help direct the course of treatment. By designing and running a series of diagnostic activity assays I hope to determine the best conditions.

III. Materials and Methods

A. Materials:

All chemicals, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO, USA). These included the membrane scaffold protein MSP1E3D1and Amberlite XAD-2 hydrophobic beads. The phospholipids 1,2-dimyristoyl-snglycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-snglycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). His Pur Ni-NTA resin was purchased from ThermoFisher Scientific (Waltham, MA, USA). The activity assays were performed using the Universal Kinase Activity Kit (R&D Systems) with an EGFR-specific substrate (Anaspec).

B. Cell culture and lysis:

Ba/F3 cells were obtained from the Nyati lab: WT Ba/F3, which is EGFR-negative as well as Ba/F3 cells engineered to express the L858R and the L858R/T790M/C797S EGFR mutants. WT Ba/F3 is IL-3 dependent so cell survival and proliferation requires addition of IL-3 (from mouse; Sigma) into the cell media (RPMI 1640 with 10% by volume Fetal Bovine Serum and 1% by volume Penicillin/Streptomycin/Glutamine; all from Fisher). Ba/F3 L858R and LTC are grown in the same cell media but without IL-3. All Ba/F3 cells were lysed by centrifuging the cells in the media at 300kx g for 5 minutes (at 4 °C), followed by washing the cell pellet in DPBS (Fisher) and the addition of approximately 1 mL per 10^8 cells of cell lysis buffer (NP40 cell lysis buffer (Fisher) supplemented with 1 mM PMSF (Cell Signaling), protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Fisher). The cells are lysed on ice for 30 minutes with vortexing every 10 minutes, centrifuged at 13k rpm for 10 minutes (at 4 °C) and BCA assay (Fisher) is performed for total protein content before storage at -80 °C.

C. Bulk Preparation

Library Nanodiscs were prepared using bulk preparation. I used phospholipids, primarily POPC, MSP1E3D1 as a scaffold protein, and Ba/F3 L858R as the primary lysate. These are combined in an Standard Disc Buffer (SDB(-/-)), which is composed of 20 mM Tris HCL pH 7.4 and 100 mM NaCl, with 20 mM sodium cholate detergent and equilibrated for 30 mins to an hour at 4 C. At this point, the detergent removal Amerlite beads are added, and left to rotate overnight at 4 C. The next day the beads are filtered out leaving formed Nanodiscs.

D. Ni-NTA purification

Ni-NTA purification was performed using 0.2 mL Ni-NTA spin columns. The resin was prepared by washing with 400 µL Purification Buffer, containing 20 mM Tris HCL pH 7.4 and 300 mM NaCl two times, with centrifugation at 700 xg for 2 minutes to remove the buffer for each wash step. Then the samples were loaded to the beads and allowed to mix for 30 minutes at room temperature. The unbound sample is removed by centrifugation, followed by three 400 µL wash steps using the Wash Buffer, which is the Purification Buffer with 50 mM imidazole. After the wash steps, the sample is eluted using 200 µL Elution Buffer, which is the Purification Buffer with 500 mM imidazole.

E. Size-exclusion chromatography:

Nanodiscs were characterized using size-exclusion chromatography (SEC) after Bulk or microfluidic Nanodisc preparation to confirm Nanodisc formation. Samples were injected onto a Superdex 200 Increase 3.2/300 column (GE Healthcare, Pittsburgh, PA, USA) at a flow rate of

50 µL/min and absorbance was monitored at 280 nm. A mixture of Thyroglobulin, γ-globulin, Ovalbumin, Myoglobin and Vitamin B12 (BioRad, Hercules, CA, USA) are used as chromatographic standards. The running buffer used was Standard Disc Buffer (SDB – 20 mM Tris, 100 mM NaCl, 0.5 mM EDTA, 0.01% sodium azide).

F. Activity Assay

The activity assays I ran were performed with the Universal Kinase Activity Assay kit. For every assay, standards are run in duplicates and each condition is run in triplicate. This assay is designed for any kinase, such as our EGFR protein of interest. The sample of interest, usually lysate or Nanodiscs, are incubated with the EGFR specific substrate, 200 nM ATP and 2 ng/µL coupling phosphatase supplied by the Universal Kinase Activity Kit (R&D Systems) for 10 mins. Active kinases are able to phosphorylate an added substrate by converting added ATP to ADP. Then a coupling phosphatase removes inorganic phosphate from ADP to form AMP. This released phosphate is detected by adding the malachite green phosphate detection reagents. Since the amount of inorganic phosphate released by the coupling phosphatase (CD39L2) is proportional to the ADP generated, the rate of inorganic phosphate production reflects the level of kinase activity. The absorbance of the wells is measured at 620 nm after 20 minutes of incubation. As a control, we often test running the assay with and without the EGFR specific substrate, to remove background signal non-specific to the EGFR.

G. Protein concentration:

The total protein concentration of the Nanodisc samples were measured using the Qubit Protein

Assay (Fisher). For this assay we used 1 µl of our sample. We would run the prediluted BSA standards to form a standard curve. Then dilute the provided reagent using the buffer from the kit and add our sample. Concentrations were measured on a Qubit Fluorometer.

IV. Results/Discussion:

To begin our optimization of our activity assays, we created library Nanodiscs. To create these we used POPC, which is a diacylglycerol and phospholipid that is found in eukaryotic cell membranes, to form the lipid bilayer in our Nanodisc. The MSP we use is a genetically engineered protein that mimics Apolipoprotein A-1. Two of the MSP1E3D1 will encircle the phospholipid bilayer to cover the hydrophobic regions. The membrane proteins incorporated in the Nanodiscs come from Ba/F3 L858R. These are the mice leukemia cells obtained from the Nyati lab containing EGFR with the L858R mutation commonly seen in NSCLC.

To begin our optimization of our activity assays, we observed the effect of various buffers, in order to determine which could be used to purify EGFR and not interfere with activity assays. Using the Malachite green assay, the following buffers were tested both with and without the inclusion of library nanodiscs to get an understanding of potential background interference or sample activity suppression:

-Standard Disc Buffer (-/-) (SDB (-/-)) - 20 mM Tris HCl pH 7.4, 100 mM NaCl. This is the buffer Nanodiscs are prepared in most often.

-Standard Disc Buffer (SDB) - This is the SDB(-/-) with 0.1% sodium azide and 0.5 mM EDTA. It is used as the SEC running buffer, with the EDTA and sodium azide preventing any biological growth.

-Elution Buffer - This is the elution buffer for Ni-NTA purification (20 mM Tris, 300 mM NaCl), which contains 500 mM imidazole.

-Glycine buffer - 0.1M glycine pH 2.8. The low pH is used for antibody elution.

-Cell Lysis Buffer - 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM

Na3VO4, 1 µg/ml leupeptin, 1 mM PMSF. Purchased from Cell Signaling Technologies and used to lyse the cells.

SDB (-/-) was used as negative control for conditions that did not contain nanodiscs to keep the same total volume for all conditions.

	Average with Nanodiscs	Standard Deviation (SD) with Nanodiscs	Average without Nanodiscs	Standard Deviation without nanodiscs	Difference
SDB(-/-)	0.1116	0.0136836	0.0906	0.0081265	0.021
SDB	0.1237	0.0229321	0.0983333	0.0095657	0.025367
Elution	0.1124	0.0102621	0.1245333	0.0284312	-0.01213
Glycine	0.1230333	0.0145926	0.1076	0.0056321	0.015433
Cell Lysis	0.2321667	0.0143532	0.1624333	0.0138338	0.069733

Table 1: Shows standard deviation (SD) and average absorbances for each condition with and without Nanodiscs, as well as the differences in the average absorbances.

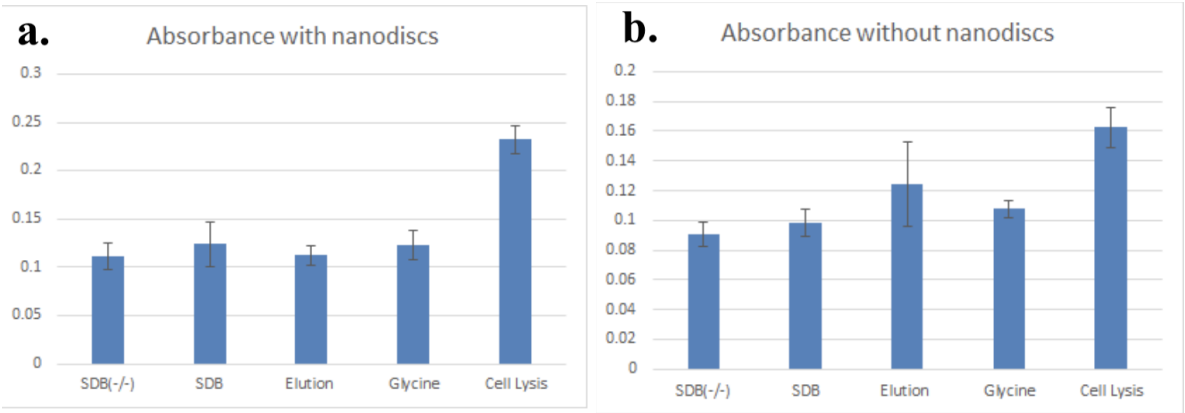


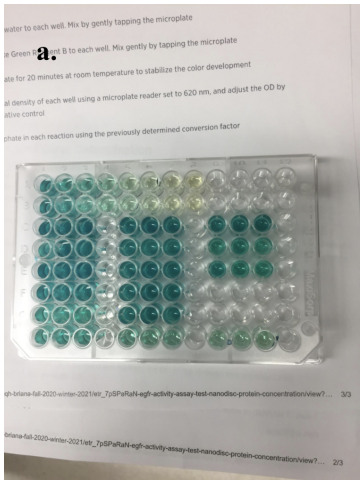
Figure 1: a. The y axis shows the absorbances of each of the buffers tested with the Nanodiscs in au (absorbance unit). Error bars are equivalent to +/- one standard deviation. The only buffer that resulted in a significantly different absorbance was the cell lysis buffer. **b.** The y axis shows the absorbances of each buffer tested without the Nanodiscs (blank samples) in au. Error bars are equivalent to +/- one standard deviation.

There did not seem to be a significant difference in the background of the samples when using the SDB(-/-), SDB, Elution, and Glycine buffers. However, the cell lysis buffer caused a higher average absorbance, even without Nanodiscs, and had the largest difference in total

absorbance between the samples with the Nanodiscs and the blanks without the Nanodiscs. The high absorbance was most likely caused by the small percentage of Triton X-100 absorbance in the cell lysis buffer, as that detergent is known to have it's own absorbance that could be interfering with this assay (**Figure 1a**).[16] The two SDB buffers had about the same change in absorbance between the samples with Nanodiscs and without, while the glycine buffer was slightly less than that. The only buffer that showed a higher absorbance in the blank was the elution buffer, but this might be due to the large error seen in the blank of this buffer (**Figure 2b)**. This was most likely due to a slight error in pipetting or the plate reader causing one of three wells to read at a 50% higher absorbance. Overall, it seems that any of these buffers other than the cell lysate buffer could be used in an activity assay without causing significant background issues that would require multiple controls or a buffer exchange step that could harm the Nanodiscs and inserted membrane proteins.

From this point, we decided to test Library Nanodiscs at concentrations of 50%, 40%, 30%, 20%, 10%, 5%, 1%, and 0% in both SDB(-/-) and Cell Lysis Buffer. The goal of this was to test background protein absorbance in the EGFR activity assay. For this assay we did not have access to a plate reader so ImageJ was used to obtain absorbance readings (**Figure 2a**).

a.



b.

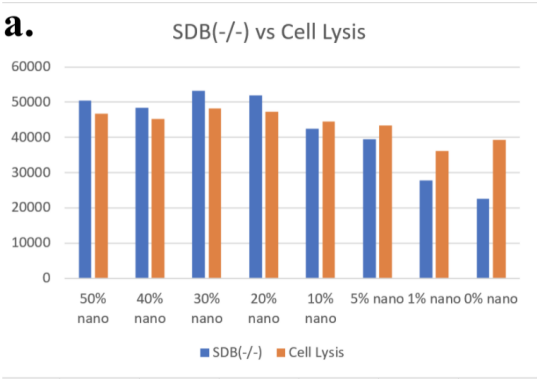
	1	2	3	4	5	6	7	8	9	10	11	12
A	standards	standards	standards	standards	standards	standards	standards	standards				
B	standards	standards	standards	standards	standards	standards	standards	standards				
C	SDB 50% Nano	SDB 50% Nano	SDB 50% Nano		Cell lysis 50% Nano	Cell lysis 50% Nano	Cell lysis 50% Nano		Cell lysis 5% Nano	Cell lysis 5% Nano	Cell lysis 5% Nano	
D	SDB 40% Nano	SDB 40% Nano	SDB 40% Nano		Cell lysis 40% Nano	Cell lysis 40% Nano	Cell lysis 40% Nano		Cell lysis 1% Nano	Cell lysis 1% Nano	Cell lysis 1% Nano	
E	SDB 30% Nano	SDB 30% Nano	SDB 30% Nano		Cell lysis 30% Nano	Cell lysis 30% Nano	Cell lysis 30% Nano		Cell lysis no Nano	Cell lysis no Nano	Cell lysis no Nano	
F	SDB 20% Nano	SDB 20% Nano	SDB 20% Nano		Cell lysis 20% Nano	Cell lysis 20% Nano	Cell lysis 20% Nano					
G	SDB 10% Nano	SDB 10% Nano	SDB 10% Nano		Cell lysis 10% Nano	Cell lysis 10% Nano	Cell lysis 10% Nano					
H	SDB 5% Nano	SDB 5% Nano	SDB 5% Nano		SDB 1% Nano	SDB 1% Nano	SDB 1% Nano		SDB no Nano	SDB no Nano	SDB no Nano	

Figure 2: a. Image used to obtain absorbance values using ImageJ. **b.** Well plate layout corresponding to image a.

	SDB(-/-\ Average Absorbance	Standard Deviation SDB (-/-)	Cell Lysis Average Absorbance	Standard Deviation Cell Lysis	Differences
50% nano	50451.9	9075.800558	46732.49	3463.069131	3719.41
40% nano	48391.41233	8877.303267	45268.719	2413.978435	3122.69333
30% nano	53284.17333	4172.487143	48302.25433	5751.91818	4981.919
20% nano	52001.32533	2245.340537	47273.975	5177.625231	4727.35033
10% nano	42402.74267	6920.256498	44480.08967	5651.446385	-2077.347
5% nano	39463.04633	2279.522463	43472.67	3201.102194	-4009.62367
1% nano	27882.66567	6478.441027	36169.00167	4090.332973	-8286.336
0% nano	22668.628	4419.708554	39276.003	3560.89899	-16607.375

Table 2: Absorbance and SD for library nanodiscs in SDB(-/-) and cell lysis buffer, as well as the difference between SDB(-/-) and cell lysis average absorbance for each concentration. SDB(-/-) had a higher SD the cell lysis.

a.



b.

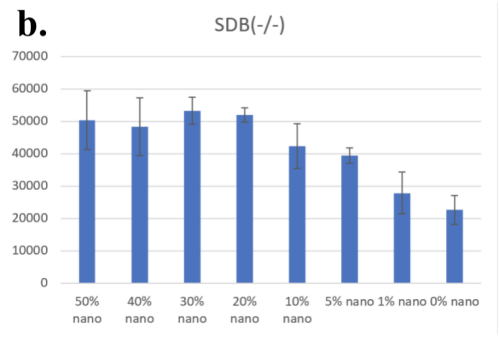


Figure 3: a. The y axis shows average absorbance of SDB(-/-) and Cell lysis for each condition in au. Cell lysis buffer averages do not vary much between different concentrations of Nanodiscs. **b.** The y axis shows the average absorbance for each concentration of Nanodiscs in SDB(-/-) in au. Error bars indicate +/- one SD.

From this attempt we still observed significant background noise when using the cell lysis buffer, as indicated by its high averages at all concentrations of Nanodiscs (**Figure 3a**). Additionally, after 20% Nanodisc concentration, the assay appears to be saturated for both buffers (**Figure 3b**). Due to the use of Image J there were concerns that there could be issues due to **Figure 2a** not containing an overhead shot and the presence of shadows. Overall, we were unsure of the accuracy of ImageJ compared to the plate reader. For this reason a modified version of this assay was run again, once the plate reader was available. For the second attempt, just SDB(-/-) and library or empty (the term for Nanodiscs not including membrane proteins, just lipids and MSP, which is another common control we used) Nanodiscs were used at the same concentrations as the previous assay.

	Library Nanodisc Average Absorbance	**Library Nanodisc Standard Deviation**	**Empty Nanodisc Average Absorbance**	**Empty Standard Deviation**	**Differences**
50% nano	1.6536667	0.0718424	0.136	0.0150997	1.5176667
40%nano	1.4073333	0.1172277	0.4936667	0.2316513	0.9136666
30% nano	1.066	0.056205	0.1126667	0.0117189	0.9533333
20% nano	0.7606667	0.1630716	0.112	0.0055678	0.6486667
10% nano	0.5343333	0.0754476	0.2916667	0.0870077	0.2426666
5% nano	0.335	0.0138924	0.1606667	0.0015275	0.1743333
0% nano	0.293	0.1189496			

Table 3: The average absorption and standard deviation of library and empty Nanodiscs at tested concentrations, as well as the difference between each of the readings for empty and library.

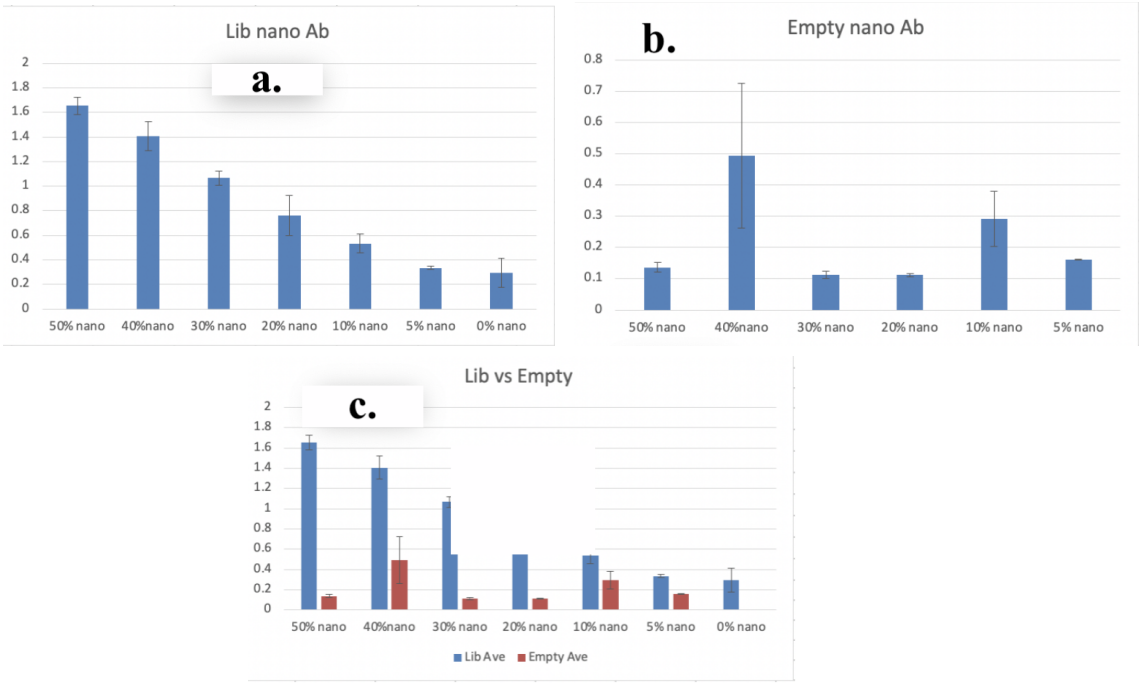


Figure 4: a. The y axis shows average absorbance for each concentration of library Nanodiscs. Error bars indicate +/- one SD. **b.** The y axis shows the average absorbance for each concentration of empty Nanodiscs. Error bars indicate +/- one SD. There is more variation in absorbances than expected but overall it shows a low absorbance. **c.** The y axis shows the average absorbance for library and empty Nanodiscs at each concentration. Error bars indicate +/- one SD. The difference in each condition between library and empty Nanodiscs is significant.

Despite the error seen in the empty nanodisc condition, there was still a clear difference between the absorbance in each condition. In the library nanodisc, we see the absorbance continues to increase until 50% Nanodisc concentration, which goes against our previous data that showed it plateaued around 20% Nanodisc concentration (**Figure 4a**). This indicates that obtaining absorbance from an image gave inaccurate results, indicating the use of a plate reader to measure the true absorbance values is required.

Since these early activity assays showed significant amounts of background noise caused by remaining lysate components, we decided that Nanodisc purifications were necessary prior to the activity assay measurement. Nickel-nitriloacetic acid (Ni-NTA) column chromatography is used to purify only anything containing the MSP1E3D1 used for the Nanodisc formation, which is engineered with a 6-histidine tag that binds to the Ni-NTA. This includes both successfully

formed Nanodiscs, with or without membrane proteins incorporated, and any remaining MSP that did not form Nanodiscs. Any components that do not contain the His-tag will be eluted in wash steps of the column chromatography while MSP will remain bound until the imidazole-containing buffer is used in the elution steps.

The next step was to test purified and unpurified Nanodiscs in SDB(-/-) at various Nanodisc concentrations at or below a 50% concentration. For our first test we ran unpurified Nanodiscs, purified Nanodiscs, and the loading step from the Ni-NTA purification at concentrations of 50%, 30%, 10%, 0% nanodiscs.

	Library Nanodisc Average Absorbance	**Library Nanodisc Standard Deviation**	**Purified Nanodisc Average Absorbance**	**purified Nanodisc Standard Deviation**	**Loading Step Average Absorbance**	**Loading Step Standard Deviation**
50% nano	1.4936667	0.2646324	0.1158333	0.003656	1.1573333	0.059792
30% nano	1.2643333	0.0898016	0.1338333	0.0041191	0.8116667	0.064677
10% nano	0.6216667	0.0205994	0.1931667	0.0193123	0.5913333	0.065151
0% nano	0.3293333	0.0544549				

Table 4: Average absorbance and standard deviation of each condition

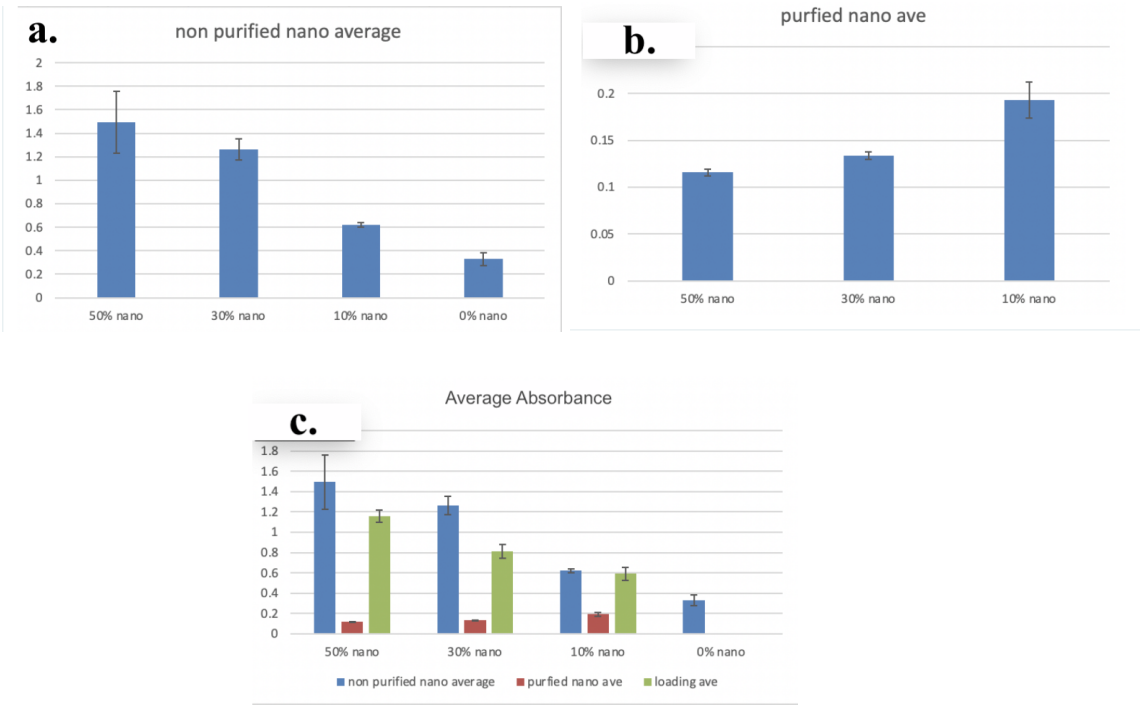


Figure 5: a. The y axis shows the non-purified (library) Nanodisc absorption in au. Error bars indicate +/- one SD. The general trend and absorbances are similar to what would be expected based on results from Figure 4. **b.** The y axis shows the purified Nanodisc average absorption in au. Error bars indicate +/- one SD. Due to error, absorbances are generally low overall, but the absorbance did increase as the concentration decreased. **c.** The y axis indicates average absorptions for each condition in au. Error bars show +/- one SD. It is clear at all concentrations that purified Nanodiscs have a significantly lower absorption than non-purified Nanodiscs.

From this data we were able to conclude that the majority of the activity that has been measured in the activity assay has likely been coming from background lysate rather than Nanodiscs. This can be seen from our purified nanodiscs having almost no readable activity and our Ni-NTA loading step, which would contain most of the background lysate, having activity levels similar to our non-purified nanodiscs (**Figure 5c**). Based on this data the next step we decided to take was to use 10 kDa molecular weight cut-off spin-column to get a higher concentration of the purified Nanodiscs in order to observe EGFR specific activity.

For this we included the unpurified (library) Nanodiscs, purified and concentrated Nanodiscs, the Ni-NTA loading step elution, and a Nanodisc-free blank. Each one was run with and without the EGFR substrate to test for non-specific activity.

	Library Nanodiscs w/ Substrate	Library Nanodiscs w/out Substrate	Purified Nanodiscs w/ Substrate	Purified Nanodiscs w/out Substrate	Loading Step w/ Substrate	Loading Step w/out Substrate	No Nanodiscs
Average	0.7091	0.64223333	0.14053333	0.1175	0.5188	0.4234	0.1412
SD	0.0666901	0.0676695 2	0.026495 53	0.0114590 6	0.0301615 6	0.0228527 9	0.0090316 1

Table 5: Average absorbance and standard deviation for each condition

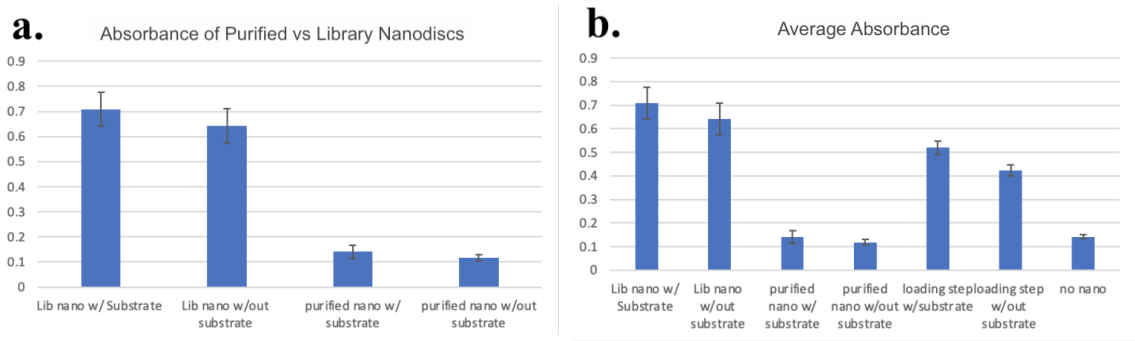


Figure 6: a. The y axis shows the average absorbance of library and purified Nanodiscs in au. Error bars indicate +/- one standard deviation. **b.** The y axis shows the average absorbance for each condition in au. Error bars are +/- one standard deviation. All Nanodiscs were added in at full concentration and purified nanodiscs were concentrated to get an equal protein concentration. In the full chart, it is easy to see that the absorbance of the loading step is closed to that of the unpurified Nanodisc. The purified Nanodisc absorbance is not significantly different from the absorbance from the condition without Nanodiscs.

This data further confirmed that most of the activity measured in the activity assays was from background lysate. Unfortunately, there is no significant activity seen in our purified Nanodiscs, nor a significant change with and without the substrate in any of the Nanodisc conditions (**Figure 6a**). This data could indicate that the activity measured is not coming from EGFR and we need to rethink some parts of the activity assay.

One of the potential ways to improve the activity assay results is to further purify the Nanodisc samples for EGFR specifically to remove background activity and improve the consistency between assays by having a better idea of the total EGFR activity in a sample. The idea is to use anti-EGFR antibodies to purify out only EGFR-containing Nanodiscs. While the

graduate student works on optimizing the purification, I have worked on determining whether the conditions used for these purifications would cause any problems with the activity assay.

Another advantage of purifying EGFR is that it will allow us to switch from our standard EGFR-specific substrate to a pan-kinase substrate, which I also tested out. For this we tested lysate, loading step, elution step, and biotin with Phosphate-Buffered Saline (PBS) with and without the pan-kinase substrate. The initial EGFR purification suggested using PBS as the main buffer, with biotin used to elute the EGFR, so I tested both PBS and biotin for background activity.

a.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8				
B	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8				
C												
D	Lys w/ sub	Lys w/ sub	Lys w/ sub	Load w/ sub	Load w/ sub	Load w/ sub	Elute w/ sub	Elute w/ sub	Elute w/ sub	Biotin w/ sub	Biotin w/ sub	Biotin w/ sub
E	Lys w/o sub	Lys w/o sub	Lys w/o sub	Load w/o sub	Load w/o sub	Load w/o sub	Elute w/o sub	Elute w/o sub	Elute w/o sub	Biotin w/o sub	Biotin w/o sub	Biotin w/o sub
F	Buff w/ sub	Buff w/ sub	Buff w/ sub									
G	Buff w/o sub	Buff w/o sub	Buff w/o sub									
H												

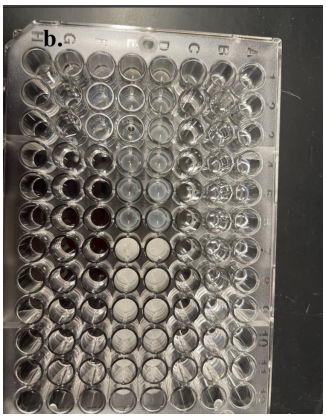


Figure 7: a. Activity Assay layout corresponding to the wells in Figure 7b. **b.** Precipitate was visible in all test wells, besides the buffer without substrate. Image was taken after the addition of Reagent A.

As seen in Figure 7b, all the samples showed at least some white precipitate formation, especially the samples of interest containing biotin. The exact cause of this precipitate was unclear. Possible causes include the biotin, the use of 1 M NaOH to solubilize the biotin, or the PBS buffer everything was washed with and diluted in. We also realized that the PBS buffer would cause issues in the final steps, as the Malachite Green reagents measure free phosphate, so we decided to test other buffers for the purification.

To determine if biotin was causing these issues, I tested a few different buffers containing biotin. Solid biotin can be solubilized in both 1 M NaOH or DMSO. In each glass vial 0.1 g of biotin was solubilized with 500 uL NaOH or 1 mL DMSO and stored at 4 C. Both the NaOH and DMSO samples were diluted as listed in Table 6 in the following three buffers : Tris-based SDB(-/-) buffer, HEPES based purification buffer, and activity assay buffer that comes with the activity assay kit (AA). In order to avoid wasting reagents, only Malachite Green A reagent, water, and then the Malachite Green B reagent were used at the usual ratios. The formation of precipitate was documented for upon combination of the NaOH/DMSO and buffer, as well as upon the addition of Reagent A.

	Stock (uL)	Buffer (uL)
50%	100	100
25%	50	150
10%	20	180
5%	10	190
2.5%	5	195
1%	2	198
0.1%	0.2	199.8
0	0	200

Table 6: Amount of stock NaOH/DMSO with biotin and buffer adder to achieve the correct concentration of stock solution.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NaOH/Tris 50%	NaOH/Tris 50%	NaOH/Tris 50%	NaOH/Tris 25%	NaOH/Tris 25%	NaOH/Tris 25%	NaOH/Tris 10%	NaOH/Tris 10%	NaOH/Tris 10%	NaOH/Tris 5%	NaOH/Tris 5%	NaOH/Tris 5%
B	NaOH/Tris 2.5%	NaOH/Tris 2.5%	NaOH/Tris 2.5%	NaOH/Tris 1%	NaOH/Tris 1%	NaOH/Tris 1%	NaOH/Tris 0.1%	NaOH/Tris 0.1%	NaOH/Tris 0.1%	NaOH/Tris 0%	NaOH/Tris 0%	NaOH/Tris 0%
C	NaOH/HEPES 50%	NaOH/HEPES 50%	NaOH/HEPES 50%	NaOH/HEPES 25%	NaOH/HEPES 25%	NaOH/HEPES 25%	NaOH/HEPES 10%	NaOH/HEPES 10%	NaOH/HEPES 10%	NaOH/HEPES 5%	NaOH/HEPES 5%	NaOH/HEPES 5%
D	NaOH/HEPES 2.5%	NaOH/HEPES 2.5%	NaOH/HEPES 2.5%	NaOH/HEPES 1%	NaOH/HEPES 1%	NaOH/HEPES 1%	NaOH/HEPES 0.1%	NaOH/HEPES 0.1%	NaOH/HEPES 0.1%	NaOH/HEPES 0%	NaOH/HEPES 0%	NaOH/HEPES 0%
E	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A	NaOH/A

	A 50%	A 50%	A 50%	A 25%	A 25%	A 25%	A 10%	A 10%	A 10%	A 5%	A 5%	A 5%
F	NaOH/A A 2.5%	NaOH/A A 2.5%	NaOH/A A 2.5%	NaOH/A A 1%	NaOH/A A 1%	NaOH/A A 1%	NaOH/A A 0.1%	NaOH/A A 0.1%	NaOH/A A 0.1%	NaOH/A A 0%	NaOH/A A 0%	NaOH/A A 0%

Table 7: Layout for NaOH assay. Wells labeled in green had visible precipitate upon the addition of Reagent A.

	1	2	3	4	5	6	7	8	9	10	11	12
C	DMSO/ Tris 50 %	DMSO/ Tris 50 %	DMSO/ Tris 50 %	DMSO/ Tris 25 %	DMSO/ Tris 25 %	DMSO/ Tris 25 %	DMSO/ Tris 10 %	DMSO/ Tris 10 %	DMSO/ Tris 10 %	DMSO/ Tris 5%	DMSO/ Tris 5%	DMSO/ Tris 5%
D	DMSO/ Tris 2.5 %	DMSO/ Tris 2.5 %	DMSO/ Tris 2.5 %	DMSO/ Tris 1%	DMSO/ Tris 1%	DMSO/ Tris 1%	DMSO/ Tris 0.1 %	DMSO/ Tris 0.1 %	DMSO/ Tris 0.1 %	DMSO/ Tris 0%	DMSO/ Tris 0%	DMSO/ Tris 0%
E	DMSO/ HEPES 50 %	DMSO/ HEPES 50 %	DMSO/ HEPES 50 %	DMSO/ HEPES 25 %	DMSO/ HEPES 25 %	DMSO/ HEPES 25 %	DMSO/ HEPES 10 %	DMSO/ HEPES 10 %	DMSO/ HEPES 10 %	DMSO/ HEPES 5%	DMSO/ HEPES 5%	DMSO/ HEPES 5%
F	DMSO/ HEPES 2.5 %	DMSO/ HEPES 2.5 %	DMSO/ HEPES 2.5 %	DMSO/ HEPES 1%	DMSO/ HEPES 1%	DMSO/ HEPES 1%	DMSO/ HEPES 0.1 %	DMSO/ HEPES 0.1 %	DMSO/ HEPES 0.1 %	DMSO/ HEPES 0%	DMSO/ HEPES 0%	DMSO/ HEPES 0%
G	DMSO/ AA 50 %	DMSO/ AA 50 %	DMSO/ AA 50 %	DMSO/ AA 25 %	DMSO/ AA 25 %	DMSO/ AA 25 %	DMSO/ AA 10 %	DMSO/ AA 10 %	DMSO/ AA 10 %	DMSO/ AA 5%	DMSO/ AA 5%	DMSO/ AA 5%
H	DMSO/ AA 2.5 %	DMSO/ AA 2.5 %	DMSO/ AA 2.5 %	DMSO/ AA 1%	DMSO/ AA 1%	DMSO/ AA 1%	DMSO/ AA 0.1 %	DMSO/ AA 0.1 %	DMSO/ AA 0.1 %	DMSO/ AA 0%	DMSO/ AA 0%	DMSO/ AA 0%

Table 8: Layout for DMSO assay. Blue indicates wells that had precipitate formed before the addition of Reagent A from the activity assay kit.

NaOH led to precipitate upon the addition of Malachite Green Reagent A, indicating that the precipitate may have been caused by the basicity of the solution causing issues with that reagent (**Table 7**). On the other hand, the DMSO conditions caused precipitate formation upon

combination with the buffer. The biotin is likely not the issue in this case, due to the lack of precipitate in the NaOH conditions before addition of Reagent A (**Table 8**). This could indicate that this is a new issue that is caused by DMSO itself and diluted NaOH might be the better solution to be used to solubilize the biotin. The precipitation in the initial assay we believe might have been caused by the new substrate tested, possibly because of the buffer the substrate was stored in.

While this work is still ongoing and the activity assays have not been fully optimized to show the activity of EGFR specifically, some conclusions can still be drawn. Of the original five buffers tested SDB(-/-), SDB, Elution, and Glycine buffers are all acceptable to use in activity assays, with SDB(-/-) being the default. From this we were able to show that the activity we can currently see stailizes at around 50% Nanodisc concentration, which will reduce the amount of sample needed in the future. Unfortunately, we were not able to observe activity from our purified nanodiscs at any concentration. This indicates that we may need to try different purification or activity assay conditions. In our attempt to switch the EGFR substrates, as well as try out other buffers, we had precipitate formation. We concluded that the issue could be involved in the PBS, NaOH, or the biotin involved in this assay. Further tests, relieved that both NaOH and DMSO will form precipitate in our assays, though at different points in the assay. Future work will still be needed to determine the best purification methods based on this data.

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