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1 Abstract:

Ingenuity Lab's artificial photosynthesis process is a multi-enzyme platform inspired by nature. A nonliving system mimicking photosynthesis in plants, it captures industrial CO_2 emissions along with sunlight and water to generate value-added, organic chemicals. The process consists of a bioreactor cascade that allows for optimized reaction conditions, overcoming the limitations and inefficiencies that are inherent in traditional biotechnology systems. By using CO_2, our technology not only reduces greenhouse gas emissions, but it generates revenue. Ingenuity Lab is engineering a potentially revolutionizing suite of nature-inspired, breakthrough technologies to convert CO2 emissions into value-added chemicals by mimicking plant photosynthesis. This technology uses only CO2, light, and water as feedstock in a cascade of bioreactors optimized for superior reaction kinetics, separation, and product yields. The enzymes are immobilized, significantly increasing their stability and reusability and consequently, simplifying product separation. The energy required for the conversion of CO2 into organic molecules is supplied by co-factor molecules ATP regenerated through proteins embedded in nanovesicle polymerosomes.and NADH regenerated through electrochemical reaction.

Keywords: Artificial photosynthesis; Bioreactor; CO_2 emission; and Value-added chemicals

2 Executive Summary

Over the last several years, the use of biotechnology for sequestering carbon dioxide (CO2) has gained considerable attention. Although these technologies have been shown to have a significant reduction in CO2 emissions, there are inefficiencies associated with using whole cell biocatalysts. The use of living organisms is energy and nutrient intensive as these inputs are wasted on mechanisms for cellular growth and repair that do not directly result in the production of targeted compounds. Additionally, industrial chemical manufacturing requires energy and extensive downstream processing to create marketable products, thus further reducing the potential for CO2 emission reduction. Our approach focuses on overcoming the limitations and inefficiencies that are inherent in traditional biotechnology systems used for carbon sequestration.

The technology is a multi-enzyme platform that generates valuable, small organic molecules from CO2 produced by industrial processes, sunlight, water, and electricity. It essentially provides photosynthesis without the energy requirements for reproduction and growth found in traditional biological carbon fixation platforms. The process is designed as a cascade of bioreactors, which allow for optimized reaction conditions at each stage of the process.

The main objective of this project is to further develop and optimize a scalable system that utilizes GHG emissions for the generation of valuable, directly marketable small molecules.

To achieve our goal which was mimicking photosynthesis process in ex-vivo condition, (a) we identified the most economical source of enzymes for the application in vitro of the enzymatic cycle that catalyzes the photosynthetic assimilation of carbon dioxide by the Calvin Cycle. The commercial availability of the enzymes for this project is essentially limited to conventional biochemical laboratory suppliers which renders our process economically impractical. For this reason, and to develop critical supply-chain independence, our research team developed a set of methodologies to produce and to purify all the enzymes in the Calvin Cycle in-house at lab scale. This is accomplished through genetically engineering E-coli to express appropriate genes for the production of each enzyme. Currently, Ingenuity Lab is working with the third party to scale-up

the production of these enzymes. A significant amount of effort was put forth in the research for the best selection of the isozymes produced in this project. The expression and purification were developed with the anticipation of scaling-up the production of the enzymes. With this in mind, each process was designed to generate the desired product, at acceptable purity levels with minimal steps and with high yields; (b) we created a database of comprehensive kinetic rate equations for each enzyme involved in the Calvin Cycle to have the required information for process design; (c) we designed a formulation of the ranaspumin (RSN) peptides demonstrating greatest ability to impact the process positively; (d) we developed promising methodologies for ATP regeneration system using proteopolymersome nanovesicles; (e) We developped promissing methodologies for NADH regeneration through electrochemical pathways; (f) We could be able to entrapped enzyme in tangentional hollow fiber membran; however, the efficiency of enzymes in hollow fiber membranes were almost 10% of their efficiency in the free forms; and (g) we integrated all required compartments for having the mimic photsynthesis process and we show that all the invovled compartments in the final system working although the rate was slower than expected due to the function of immobilized enzyme.
Since we entered to nrg cosia Carbon Xprize competition and Ingenuity Lab became semifinalist for this project, we have planned our efforts to scale up this project based on the defined milestones by Xprize team. The main near future milestone that we have to build the system that can capture and convert 60 kg of CO2 out of flue gas stream from natural gas power.
This synthetic Calvin Cycle engineered by Ingenuity Lab has the potential to produce almost 50 high value chemicals. These can be marketed as such or transformed into higher value commodities with available technologies.

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3 Introduction and Project Overview

The solution to the world's carbon challenge seems daunting; as the world grows, so does the demand for fossil fuels. The time for creative solutions and bold ideas that make significant and verifiable GHG reductions is now. Industrial biotechnology is used to produce biofuels, pharmaceuticals, chemicals and other products as well as for wastewater treatment and soil remediation. Over the last several years, the use of biotechnology for sequestering carbon dioxide has gained considerable attention. Although these technologies have been shown to have a significant reduction in CO_2 emissions, there are inefficiencies associated with using whole cell biocatalysts. The use of living organisms is energy and nutrient intensive as these inputs are wasted on mechanisms for cellular growth and repair that do not directly result in the production of targeted compounds. Additionally, industrial chemical manufacturing requires energy extensive downstream processing to create marketable products, thus further reducing the potential for CO_2 emission reduction. Our approach focuses on overcoming the limitations and inefficiencies that are inherent in traditional biotechnology systems used for carbon sequestration.
This project incorporates the metabolic processes responsible for carbon fixation in living organisms, i.e. photosynthesis, into a durable, self-sustaining, continuous industrial process that is engineered to create value-added chemicals by utilizing carbon dioxide as its principal

feedstock. This project thus far has developed and is demonstrating a suite of technologies that overcomes the many technical challenges that have historically rendered this approach impossible.

Our team's coordinated efforts are focused on the demonstration of a multi-enzyme platform that generates valuable, small organic molecules from CO_2 produced by industrial processes, sunlight, and water. It replicates photosynthesis in an ex-vivo system that does not sink energy into the reproduction and growth of cells. The process consists of a cascade of bioreactors that allow for optimized reaction conditions for each stage of the process. The enzymes used are immobilized to increase their stability and lifespan, and to simplify separations. Critically, the reactor vessels utilize an enabling, patented nanoscale bubble architecture [1], that has been shown to double enzymatic conversion rates.

The significant advantage of our technology is its elaborate flexibility. As there are multiple intermediate organic compounds involved in the multi-enzymatic platform, there is a superior flexibility regarding the final product of this carbon fixation technology; therefore, our technology can conform to accommodate for various economic and technical considerations.

Nature inspires the approach we adopt in this project. Plants use CO_2 and sunlight in a process commonly known as "photosynthesis" to produce organic matter. We aim to mimic the same principles in a non-living system, which we term "artificial photosynthesis," shown schematically in Figure 1.

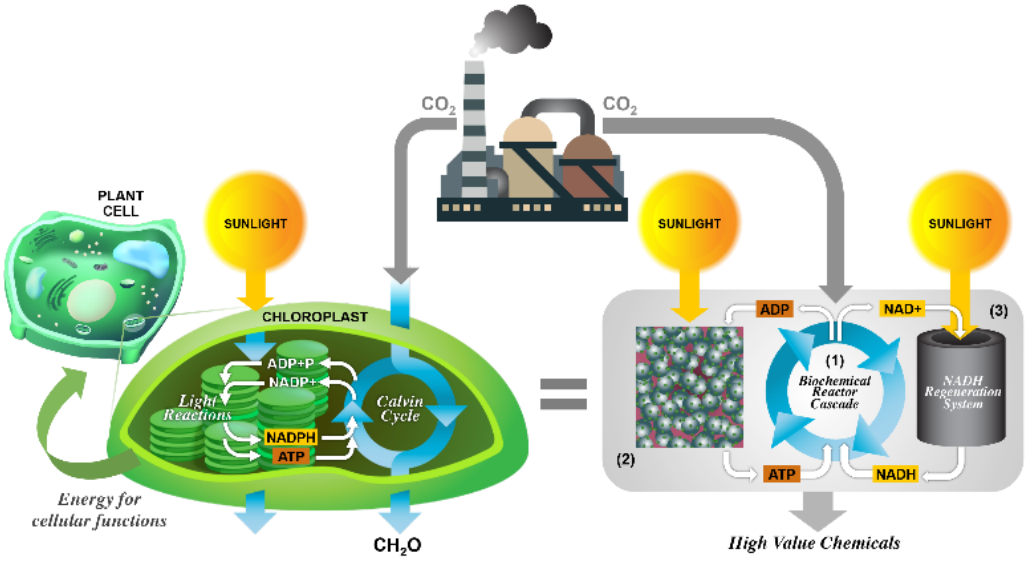

Figure 1- A schematic of artificial photosynthesis mirroring plant cell photosynthesis: (1) Biochemical reactor cascade; (2), ATP regeneration system; (3), NADH regeneration system

The remarkable ability of a plant cell to capture CO_2 and synthesize organic molecules by harnessing sunlight emerges from chloroplasts. The chloroplast is essentially the most elegant implementation of nanoscale processes by nature – there are about half a million chloroplasts in $1mm^2$ surface area of a plant leaf.

Photosynthesis consists of two main processes known as the light reactions and the light independent reaction, i.e. the Calvin Cycle. Through light reactions, the solar energy is converted

into chemical energy. Light absorbed by chlorophyll drives the transfer of electrons and a hydrogen ion to an acceptor called $NADP^+$ (nicotinamide adenine dinucleotide phosphate) to produce NADPH. The light reactions also turn ADP to ATP in a process called photophosphorylation.

The Calvin Cycle uses the chemical energy of ATP and NADPH to reduce CO_2 into organic matter. It consists of an elaborate cyclic cascade of multiple enzymes, which converts CO_2 into organic matter (Figure 2).

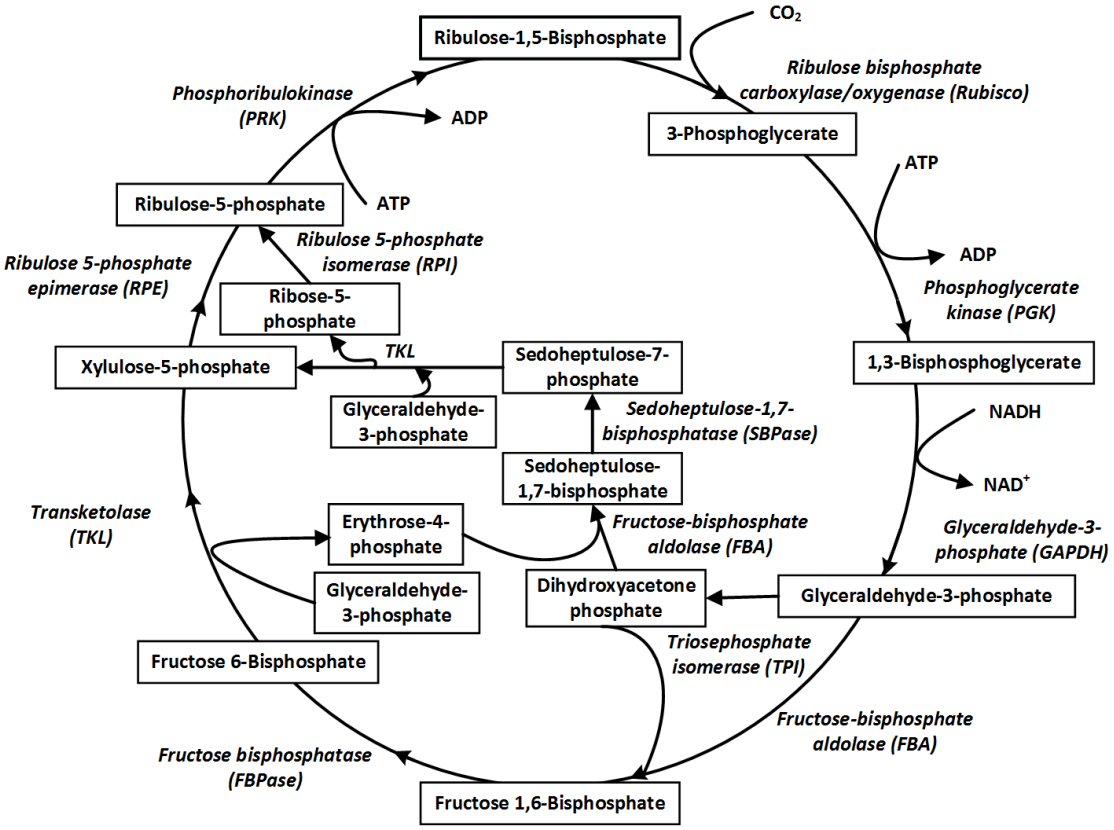

Figure 2- Calvin Cycle, the driving engine of photosynthesis and our process

The conversion of organic matter to CO_2, i.e. oxidation, releases energy and is thermodynamically, a spontaneous process. The reverse, conversion of CO_2 to organic matter, i.e. reduction, is not energetically favorable and requires energy to proceed. In photosynthesis, this energy is supplied by sunlight through the high-energy molecules NADPH and ATP. Once they release their energy, they are converted to their "low energy" or oxidized forms of $NADP^+$ and ADP, respectively. In a plant, $NADP^+$ and ADP are converted back to NADPH and ATP through light reactions as mentioned before, which occur in enzymatic complexes of photosystems I and II.

There are quite a few intermediate products in the Calvin Cycle each of which can be selectively removed from the cycle and further processed toward the desired final product. Such further processing is similarly enzymatic in nature. Therefore, a wide array of products can be produced through this technology suiting various market realities and various considerations.

The biotechnological process of Ingenuity Lab's technology involves four key elements:

(a) Adapted Calvin Cycle and Biochemical Transformation **-** The Calvin Cycle with ten enzymes, also commonly called the *reductive pentose phosphate pathway* is an enzymatic cycle that catalyzes the photosynthetic assimilation of CO_2 and produces pentoses. Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) in the cycle, the only enzyme capable of CO_2 assimilation, is the first enzyme in the cycle and catalyzes the fixation of atmospheric CO_2 to Ribulose 1,5-bisphosphate (RuBP). By employing RuBisCO's ability to sequester atmospheric CO2 and the Calvin Cycle's ability to regenerate the CO2 acceptor our bio-process converts CO2 emissions into valuable organics without bio or petro-based feedstock.

(b) Light-Driven ATP Synthesis **–** The synthesis of triose phosphate from CO2 by the Calvin Cycle requires energy in the form of adenosine triphosphate (ATP); ATP turns into adenosine triphosphate (ADP) after releasing its energy. For every three molecules of CO2 sequestered nine ATP molecules are required. In nature, ATP for CO2 assimilation is provided and cyclically regenerated by light-dependent reactions of photosynthesis in the chloroplast. Although ATP is available for purchase, it is very expensive commercially. Ingenuity Lab has developed the technology and know-how to produce artificial organelles, which mimic the ATP synthesis mechanism in chloroplasts. ATP regeneration is accomplished by employing bacteriorhodopsin-F1F0 ATP synthase proteo-polymersomes (BR-ATPase PPs) (Figure 3) [2]. In a paper published by members of Ingenuity Lab, it was demonstrated that the BR-ATPase PPs have a quantum efficiency of 96% of converting light energy into chemical energy in the form of glucose with use of the sugar producing portion of the Calvin Cycle [3]. For the production of polymerosome vesicles, Ingenuity Lab has optimized the production of the ABA (PMOXA-PDMS-PMOXA) triblock copolymer. These PDMS-base amphiphilic polymers can maintain good self-assembly properties in aqueous solution due to its inherent ability to form vesicular structures and remain stable for long periods in a wide range of conditions. Therefore, the triblock copolymers are being used as alternate to the phospholipid membrane of living cells.

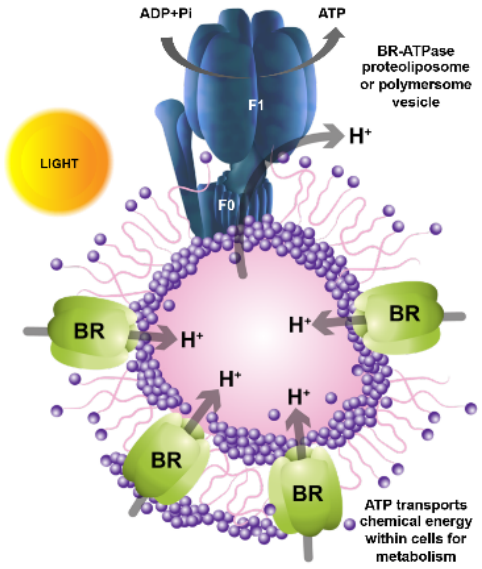

Figure 3- BR-ATPase proteoliposome vesicle

(c) Regeneration of NADH - The reducing power required by the Calvin Cycle is supplied in the form of the enzymatic co-factor Nicotinamide adenine dinucleotide (NADH). The cost of NADH remains the primary barrier to widespread usage for industrial purposes. For this reason, a large effort has been devoted to the development of NADH regeneration methods that enables the re-use of NADH initially introduced into a reactor. Ingenuity Lab developed an electrochemical methodology to regenerate NADH from NAD^+ continuously.

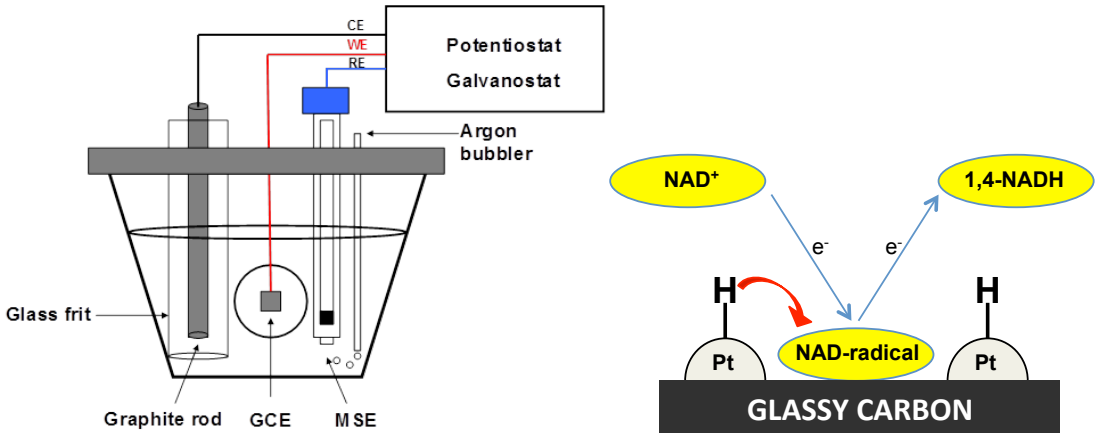

Figure 4- NADH regeneration system through electrochemical pathway

(d) Ranaspumin Foam Bubble Architecture - One of the largest challenges for the *in vitro* replication of cellular functions and metabolisms is the lack of compartmentalization and localization of biochemicals within a confined structure. To create an environment, in which

nanobiological systems can be compartmentalized to mimic nature, we developed a bubble architecture made from Ranaspumin-2 (RSN-2) [4]. RSN-2 is a protein surfactant produced by the Tungara frog to create foam nests for its fertilized eggs [5]. With the use of the RSN-2 we create foam-based biochemical reactors. This technology is superior to traditional bulk liquid bioreactors; by application of the *Law of Mass Action* molecules are concentrated in thin aqueous channels increasing interaction with one another [2]. However, we did not integrate the foam reactor in the final system because we did not want to increase the complexity of the system much more.

Ingenuity Lab defined four milestones in order to achieve the project goals. The defined milestones for the project were as follow:

(a) Foam 'Bubble Architecture" formulation
(b) Generate extensive homogenous kinetic data for process enzymes
(c) nrg cosia Carbon XPRIZE application
(d) All components in place for lab-scale reactor
(e) Immobilization of enzymes
(f) Final lab-scale system

4 Project Goals

The goal of this project is to implement, optimize a scalable engineering system that utilizes GHG emissions for the generation of drop-in high-value chemicals compatible with Alberta's petrochemical infrastructure. Discrete project deliverables over the next two years include:

- Commercial assessment of high-value chemicals that can be synthesized through biochemical transformation of process intermediates. A commercial assessment that includes all possible chemicals will be conducted. This will include: market size study, competitive analysis, pricing targets and identification of prospective industrial partners in and outside of Alberta
- Development of a novel process for the reduction of CO_2 emissions that adds additional revenue streams to the Alberta Industrial sector by generating high-value chemicals.
- To mature the technology to facilitate the transition to pilot plant scale to demonstrate the potential for significant and sustainable greenhouse gas reductions.

The primary anticipated outcome from this program is the development and commercialization of a process that converts GHGs into high value chemicals. The process will be suitable for producing a wide variety of organic molecules from polymer precursors to liquid transportation fuels to diabetic sweeteners. Additional desirable outcomes include but are not limited to the following:

- Establish Alberta as a global leader in the development and commercial deployment of nano-scale, biologically inspired, non-living systems with applications in energy and advanced materials.
- Increase direct and indirect high paying employment opportunities in Alberta, in areas such as plant operations, engineering, nanotechnology construction and administration.
- Increased revenue for electric utilities without additional capital investment.
- Direct and indirect reduction of airborne CO_2.

5 Project Report and Implementation

5.1 Generate extensive homogenous kinetic data for process enzymes

The identification of the most economical source of enzymes for the application in vitro of the enzymatic cycle that catalyzes the photosynthetic assimilation of carbon dioxide by the Calvin Cycle. The commercial availability of the enzymes for this project is essentially limited to conventional biochemical laboratory suppliers which renders our process economically impractical. For this reason, and to develop critical supply-chain independence, our research team developed a set of methodologies to produce and to purify all the enzymes in the Calvin Cycle in-house at lab scale. This is accomplished through genetically engineering E-coli to express appropriate genes for the production of each enzyme. A significant amount of effort was put forth in the research for the best selection of the isozymes produced in this project. The expression and purification were developed with the anticipation of scaling-up the production of the enzymes. With this in mind, each process was designed to generate the desired product, at acceptable purity levels with minimal steps and with high yields. Following the commercial identification of enzymes, we then produced all the required enzymes in-house using bacterial expression. Table 1 describes each enzyme, its production source and the yield.

Table 1- Calvin Cycle enzymes produced in-house

Enzyme	**Yield (mg/L)**	**Purity**	**Expression System**
RuBisCo	225	93%	*E. Coli*
PGK	9.7	97%	*E. Coli*
GAPDH	200	97%	*E. Coli*
FBAse	N/A	N/A	*E. Coli*
TPI	14.2	93.2%	*E. Coli*
F/SBPase	5.5	62%	*E. Coli*
TK	34	86.3%	*E. Coli*
RPE	65.6	65.2%	*E. Coli*
RPI	240	91.5%	*E. Coli*
XK	9.4	84.5%	*E. Coli*
PRK	33	80%	*E. Coli*
bR	13.4	98.6%	S9
ATPase	1000 U/L	N/A	PS3

The results of the efforts for this milestone is the successful development of both the skillsets and materials to successfully produce the required enzymes at favorable cost advantage over purchasing available materials from commercial suppliers. These enzymes are the key elements for the ultimate translation and commercial success of this technology. The commercial availability of the enzymes that catalyze the reactions in the Calvin cycle is limited to conventional biochemical laboratory suppliers. For this reason, and to develop critical supply-chain independence, the research team needed and developed a set of methodologies to produce and purify all the enzymes in the Calvin cycle in sufficient quantities to enable the subsequent large-scale experimentation planned in this project.

In order to have the required information for process design, we also created a database of kinetic information of in-house Calvin Cycle enzymes. The kinetic information of the produced in-house enzymes has been presented in Table 2.

Table 2- The kinetics of in-house produced Calvin Cycle enzymes

Enzyme	Substrate	Vmax uM s^{-1}	km uM	kcat s^{-1}		Ref	Literature conditions
				Exp.	Lit.		
Rubisco	RuBP	1.4e-2	19	0.65	16.6	[6]	83 °C, pH 8.3, T. koda
	CO_2	1.2e-2	3.8e3	1.1	0.25	[7]	25 °C, pH 8.3, T. koda
PGK	3PG	7.6	360	538	540	[8]	25 °C, Pseudomonas
	ATP	15.5	440	1090	519	[8]	
GAPDH	NADPH	0.107	52.5	130	70	[9]	25 °C, pH 8.7, S. aureus
TPI	G3P	6	1.29	1.02e5	6.8e4	[10]	22 °C, pH 7.6, M. tuber.
FBA	G3P/DHAP	4.5e-3	7.5	1.7	12	[11]	22 °C, pH 7.8, M. tuber.
FBPase	FBP	2.15e-4	174	0.013	0.018	[12]	30 °C, pH 7.4, M. caps.
TK	Xu5P	9.8e-5	66.7	7	5	[13]	30 °C, pH 7.6, S. cere.
RPE	Xu5P	0.13	154	328	480	[14]	Steptococcus pyogenes
RPI	R5P	313	13.4e3	2593	2100	[15]	37 °C, pH 7.6, E. coli
PRK	Ru5P	10.9	4.4e4	225	250	[16]	20 °C, pH 7, S. oler

5.2 Foam "Bubble Architecture" formulation

One of the key challenges of the development of a scalable reactor structure with adequate geometrical compartmentalization and bio-localization to effectively replicate *in vitro* the photosynthetic assimilation of carbon dioxide and production of sugar phosphates.

In a batch configuration, this problem can be solved using an aqueous foam formulated with a biocompatible surfactant protein, Ranaspumin-2 (RSN-2), the major constituent of a group of six proteins isolated from the nests of the Tungara frog *Physalaemus pustulosus*. This technology, demonstrated and patented by Dr. Montemagno [3, 17], exploits the highly confined architecture of the foam to confine substrate and enzymes in thin aqueous channels, increasing molecular interaction, and therefore realizing a higher rate of conversion compared to equivalent reactions in bulk. This configuration is particularly advantageous for biocatalyzed gas-liquid reactions because the foam structure maximizes mass transfer rates through high interfacial areas and protects the enzymes from deleterious high shear environments. The translation of this concept to a continuous process in which chemical reactions are conducted over a theoretically indefinite period of time with a steady-state supply of substrate and recovery of product requires an optimized foam formulation to procure sufficiently long-lived foams with adequate continuous liquid drainage rates.

Recombinant Ranaspumins were expressed using *E. coli* and purified according to the protocols developed by Fleming and co-workers [18].

Our bubble architecture formulation studies showed that there are two surface active Ranaspumins in the natural frog foam, RSN-2 and RSN-4. These proteins interact upon adsorption at the interface yielding a gas dispersion in the buffer phase that is more stable than the dispersions made from the single components. In particular, there exist a formulation that produces substantially more stable films with limited drainage over the two hours of experimental time scale arbitrarily selected in our study.

RSN-3, RSN-5 and RSN-6 did not show surface activity. Control experiment conducted by injecting aliquots or each of them in the subphase did not result into any measurable increase in storage shear modulus within the experimental timescale. It is possible that these proteins interact with the adsorbed layer over a much longer time period similarly to some food proteins and participate in providing long-term stability to the foam in the natural environment [19, 20].

5.3 Technoeconomic and Carbon Capture Analysis

For nrg cosia Carbon XPRIZE competition, ingenuity Lab conducted a comprehensive study to find the technoeconomical analysis of this project, process design and implantation in potential pilot –scale as well as the social, economic and environmental impact of conducting this project. A detailed concept level economic analysis was conducted for this project. The number of reactors, pumps, and separation steps were used to estimate the future capital costs using the guidelines outlined in [21]. A scenario in which the total electric energy of the system was generated through a photovoltaic (PV) system was also analyzed, in which the cost of PV panels and batteries were accounted for in the capital cost. Using a PV system eliminated the electric power costs associated with the process and reduced emissions while increasing the capital costs and land footprint; see 5. Using PV for capturing 1200 kg CO_2 per day saves $0.14 MM annually in operating costs (i.e. electricity) while increasing the capital expenditures by $4.92 MM, almost tripling the total capital cost of the project. Hence, the use of PV rendered uneconomical, and it was decided for the process will run completely on grid power.

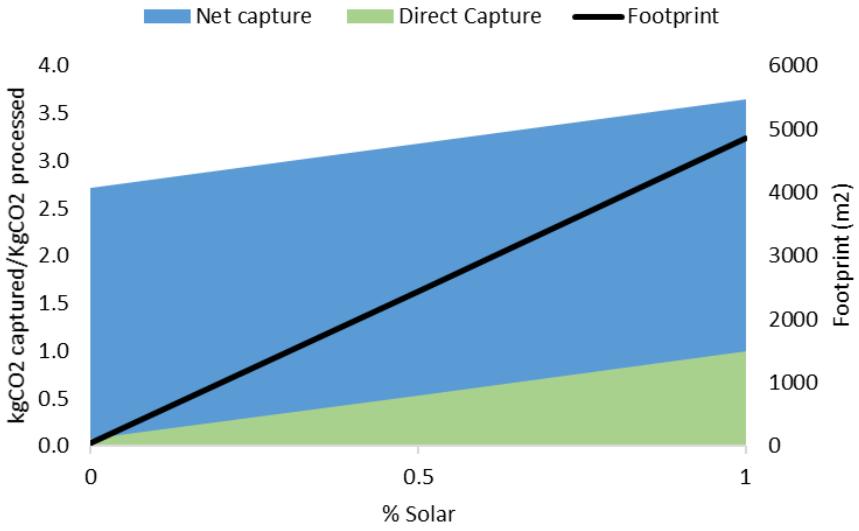

Figure 5- Effect of using PV system to power the process in terms of footprint and CO_2 (for capturing 1200 kg CO_2 per day) balance.

The direct and indirect operating costs were estimated based on methods proposed in [21]. In this analysis we have assumed dihydroxyacetone (DHA) to be the main product of the process, as it has significantly higher value per unit mass. In this case, ethylene glycol (EG) is a bonus byproduct and hence it is considered to be pure profit. Operating costs per kg DHA was estimated to be $100 for capturing 60 kg of CO2 per day and $58 for capturing 1200 kg CO2 per day. The manufacturing costs' breakdown for capturing 1200 kg CO2 per day is presented in Figure 6. It can be noticed that costs associated with enzyme use in the process dominates the economics of the process, which is typical to an enzymatic process.

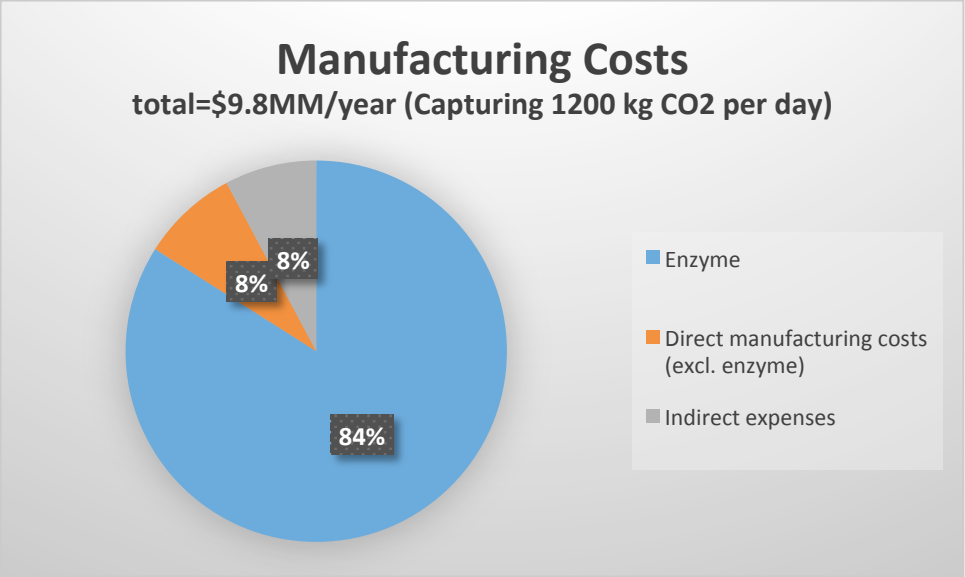

Figure 6- The major drivers of manufacturing (i.e. operating) costs

The system which will be designed to capture 60% CO2 of 200 kg and 2 tons of flue gas from natural gas power plants per day, requires a capital investment of $0.92 MM and $2.91 MM, respectively. Our analysis for capturing 1200 kg of CO2 per day indicates that the capital invested yields a <1 year investment breakeven point, a 25-year net present value of $51 MM, and a 179 % internal rate of return (IRR). The economies of scale was also studied for our process which is presented in Figure 7. It can be seen that for a conversion capacity greater than ~125 Kg CO_2 per day, the process is economically sound, e.g. positive NPV. Overall, the economic assessment of our process unveils a lucrative business case.

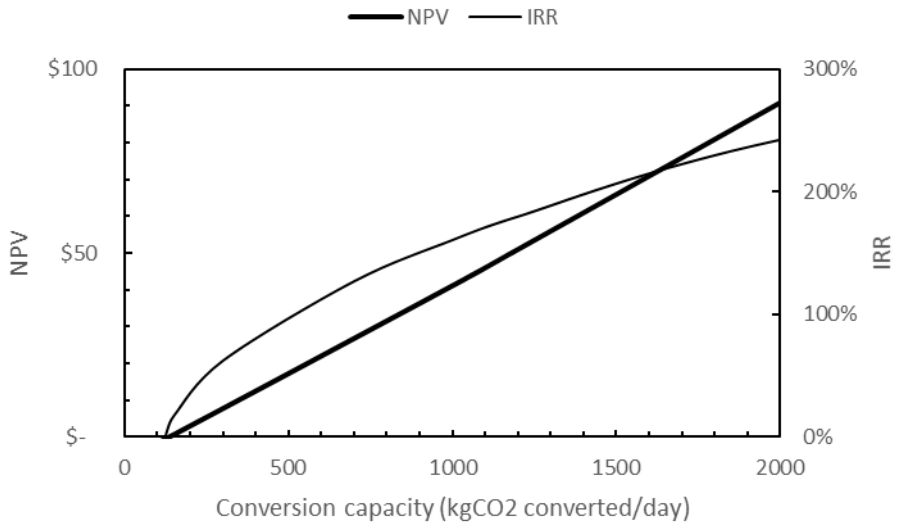

Figure 7- The effect of CO2 conversion capacity on the economic metrics of the project

5.4 NADH Regeneration System

Nicotinamide adenine dinucleotide NAD(H) is a co-enzyme involved in a large number of biochemical processes [22-28]. The purpose of NAD(H) to shuttle hydrogen and electrons. Consequently, NAD(H) is found in two redox forms: an oxidized form, NAD^+, which accepts two electrons and a hydrogen, forming an enzymatically-active reduced form, 1,4-NADH [29]. Although the potential industrial use of NAD(H) is large, due to its very high cost (especially that of 1,4-NADH) and the need to be added in a biochemical reactor in stoichiometric quantities, its current use is very limited. A solution to this problem would be to develop *in-situ* 1,4-NADH regeneration methods, and electrochemical methods are of particular interest due to their simplicity and potentially low cost [30-33].

The main objective of this part of the project in Ingenuity lab was to develop electrodes and optimize operating conditions for the *direct* electrochemical regeneration of highly-pure enzymatically-active 1,4-NADH in batch and flow electrochemical reactors. The developed electrodes will be used in a real biochemical reactor for a prolonged time to regenerate 1,4-NADH from NAD^+ during the carbon cycle to produce different enzymes. The ultimate goal is the technology commercialization of NADH regeneration from lab-scale to bench-scale and pilot-scale.

The electrochemical regeneration of NADH from NAD+ was optimized using two-compartment, three-electrode batch electrochemical reactor (cell) (Figure 4) with different types of bare electrode surfaces.

It was found that the yield of enzymatically active 1,4-NADH regenerated was highly dependent on the electrolysis potential of all the electrode surfaces employed. When bare GC was used, the yield of 1,4-NADH produced increase with an increase in regeneration potential towards more negative (cathodic) values, giving 97.45% of NADH formed at –2.30 V. We correlated the trend with an increase in surface coverage by Hads (Figure 3-2). When Ni was used, 83.14% yield of 1,4-NADH was obtained at –1.60 V, however it increased to 88.10% at –1.90 V. While on pure Ti the maximum yield (75.37 %) was obtained at –1.50 V (Table 3).

Table 3- The yield of NADH regenerated on various electrodes at different electrode potentials

Electrode	Potential / V	Yield of NADH / %
Glassy carbon	–2.30	97.45
Nickel	–1.60	83.14
Nickel	–1.90	88.10
Titanium	–1.50	75.37

5.4.1 Enzymatic reaction integrated with NADH regeneration

The compatibility of the NADH regeneration system with all components and elements involved in the reaction tested separately in different experiments before testing with its compatibility with enzymes.

The NADH regeneration system is inherently different from the rest of the process as it is an electrochemical method which involves electrodes and electric fields. We aimed to verify with

this experiment that there is no unexpected behavior (e.g. enzyme degradation or electrode malfunctions) in the system once these two processes are coupled.
The reactor mix contained all the enzymes necessary for the 3-emzyme reaction cascade presented below to occur:

$$\mathrm{RuBP} \xrightarrow{\textbf{Rubisco}} \mathrm{3PGA} \xrightarrow{ATP \longrightarrow ADP}{}^{\textbf{PGK}} \mathrm{1,3\ BPG} \xrightarrow{NADH \longrightarrow NAD^{+}}{}^{\textbf{GAPDH}} \mathrm{G3P}$$

As it can be seen in Figure 8, the initial concentration of NADH was zero, and the electrochemical regeneration reduced the NAD+ present in the reactor mix to NADH, hence the initial increase of 340nm absorption spectra. Then the electrochemical regeneration was stopped and it was allowed for the enzymes to consume the NADH, causing a decrease in NADH concentration. The cycle was repeated two more times to verify repeatability and enzyme robustness. Therefore, it has been showed that the NADH regeneration system can be integrated with enzymatic reaction.

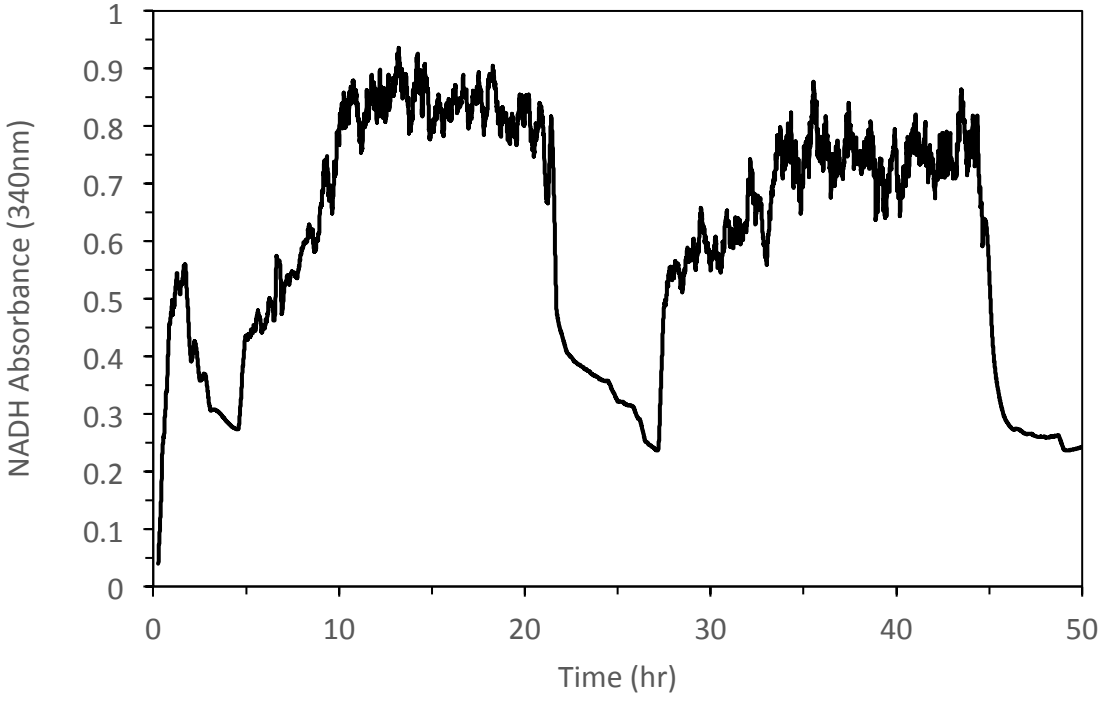

Figure 8- Enzyme reaction coupled with NADH regeneration system. Three regeneration and enzymatic oxidation can be seen.

5.5 ATP Regeneration System

ATP powers most of the energy-consuming activities of the cell and regulates many biological pathways. Much of the research has been focused on the ATP generation and mechanism since its discovery [34, 35]. The functions of cells have been mimicked through membrane proteins

and lipid membranes. Conventionally, lipid membranes and liposomes have been used for the recreation of the natural environment of membrane bound proteins. However, the future applicability of protein-reconstituted liposomes is limited due to both chemical and mechanical weaknesses. To create the artificial environment necessary for membrane bound proteins, self-assembled, amphiphilic ABA triblock copolymers have been used as a prospective building material for new biomimetic membranes.

In order to develop a protein embedded polymersome nanovesicle system for ATP regeneration in our system, poly(oxazoline) (PMOXA) is chosen as hydrophilic A block and poly(dimethylsiloxane) (PDMS) as hydrophobic B block were chosen for formation of ABA triblock system (Figure 9).

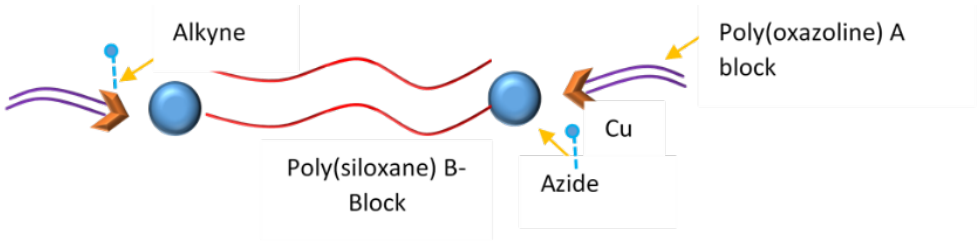

Figure 9- The A-blocks bear an alkyne terminal group, while the B-blocks are terminated with azides. The two blocks are clicked via a copper-catalyzed azide-alkyne click reaction.

Polymersomes/Vesicles were prepared through thin film rehydration method. The vesicles were characterized using Dynamic Light Scattering (DLS) (Figure 10) and Transmission Electron Microscopy (TEM).

Hydrodynamic size and polydispersity index (PDI) of the vesicles were determined using Malvern Nano-ZS DLS. Samples were measured at 25 ^{o}C at concentrations of 1 mg/ml and all measurements were performed in duplicates. The average diameter of the solution was 135 nm with PDI~0.24 when filter size was 400 nm. For filter pore size of 200 nm, the average diameter was 110 nm with PDI~0.17.

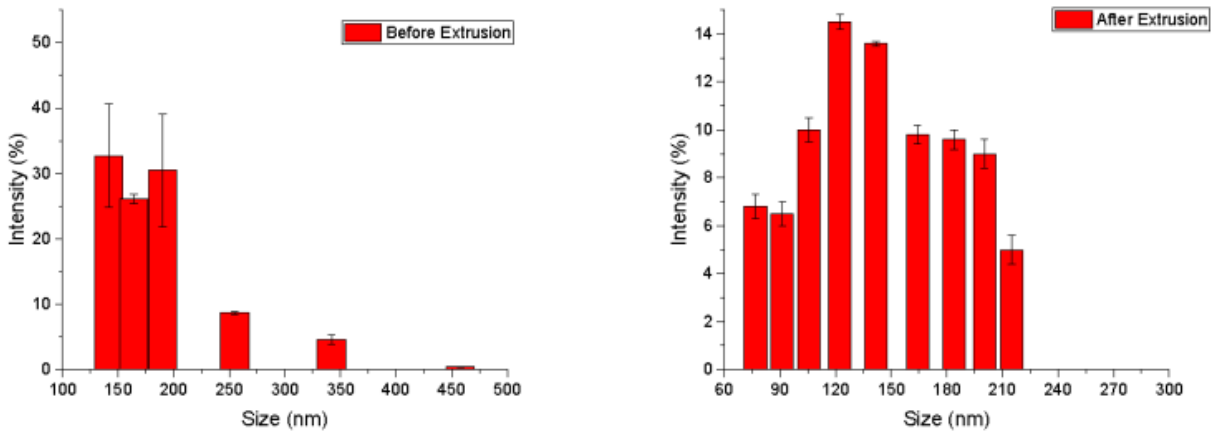

Figure 10- DLS size distribution of vesicles before and after extrusion

The morphology of the vesicles was determined using Hitachi S-4800 Field Emission SEM. For sample preparation, a drop of vesicle solution was allowed to settle on carbon coated grid. The excess of liquid was removed by gentle blotting with filter paper and drop staining solution, uranyl acetate was allowed to contact the grid for 1min. The excess of liquid was removed and the TEM grid was allowed to dry under vacuum for at least 3hrs.

We also optimized the incorporation of bacteriorhodopsin (bR) and ATPase proteins as well as their ratios to the amount of polymer. In our system, ATP has been produced by coupled reactions between bacteriorhodopsin, and F_0F_1-ATP synthase protein, reconstituted in polymersomes. ATP synthase is a rotary motor protein which is composed of two domains, F_0 integrated in the membrane and the soluble F_1. The F_0 functions to conduct protons across the membrane by a rotational mechanism of the intramembrane subunits (also called rotor), resulting in a torque which is transmitted to the catalytic sites through the rotor stalk. This mechanical energy is used by F_1 for the catalytic activity driving the synthesis and hydrolysis of ATP. The presence of light drives the bR to pump proton into the membrane and the coupling activity between the F_0 and F_1 complexes drives proton movement toward the F_1 side of the membrane, resulting in ATP synthesis [36].

To measure ATP synthesis activity, the samples were illuminated for 10min to generate a pH gradient; at which point ATP synthesis was initiated by addition of 4mM Mg-ADP and 20mM Pi. At various time point, 10µL of reaction mixture was mixed with equal volume of 4% trichloroacetic acid. The ATP generated was measured by Sigma FLAA Bioluminescent assay kit and plotted as shown in Figure 11. ATP generated from the reactions was compared with standard ATP curves determined from protocol in assay kit.

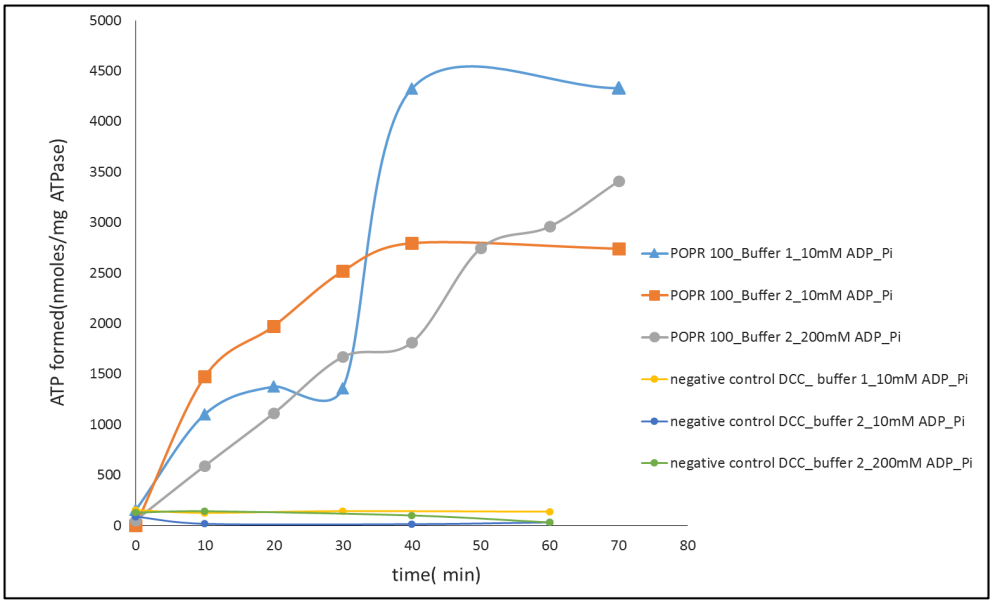

Figure 11- ATP synthesis for fixed PoPr (Polymer Protein ratio), OG concentration at 0.2 M ADP/ 0.2 M Pi as well as 10 mM ADP/10 mM Pi initiation in different buffers

5.6 Immobilization of Calvin Cycle Enzymes

Four separate strategies were explored for protein immobilization. These included layer entrapment onto frits, ABA block copolymer polymersome encapsulation, hydrogel entrapment and tangential flow membrane entrapment. The hydrogel entrapment, while providing a simple means of reaction, resulted in alcoholic bi-products during the reaction that resulted in protein denaturation.

Despite the promising results the frit immobilization data produced, its fallback results from the difficulty in quantifying the amount of deposited enzyme. Due to the nature of the spin layering technique to create a uniformly coated surface, much of the protein solution is pushed off the frit due to the centrifugal force and removed in the vacuum. Attempts to dissolve the layers and quantify the protein resulted in highly variable results. It was unclear if the inconsistencies were due to the deposition technique, the re-solubilization step, or the error in measurement due to the very low amount of protein deposited. Ten layers of 4 µl drops containing 2 mg/ml of protein would result in a maximum of 80 µg of protein deposited. While this is a tremendous amount of enzyme for kinetic studies, it is far below the detectable threshold of the Direct-Detect (in-house IR measuring device) and the use of polystyrene generates significant background absorption for UV measurements at 280 nm. Given that we had no clear way to accurately determine the amount of protein deposited using this strategy and that previous experiments determined that precise amounts of protein would be required for cycle manipulation, this method was abandoned in favor of other techniques.

Hollow fiber filter modules were used for immobilization of enzymes in which the hollow fiber filter module system was very quantifiable, easy to scale up and provided the luxury of working with soluble enzyme as is without any manipulation. To further develop the proper ratios, we explored both a 1:1 and a 2:1 successive enzyme load. These results confirmed that in a cyclical

system that increasing quantities of enzyme would be needed to drive the reaction. The downside to this, is that the amounts of FBA, FBPase and TK required are orders of magnitude greater than that of Rubisco. In order to produce a reactor capable of keeping up with the regeneration of NADH (1 umole/min), it was determined that we would need to construct a reactor with 40 mg of Rubisco and scale the remaining enzymes relative to the 2x values reported in table 2. This would equate to requiring in excess of 244 mg of FBA, 462 mg of FBPase and 237 mg of TK. On hand quantities of these enzymes were significantly less, thus we chose to construct a reactor relative to the amount of active FBA we had (23 mg). This should produce a reactor capable of turning over 50 nmoles of RuBP per minute and would scale up the reactor 1000 times larger than then experimental free state reactions used in the kinetic analysis. The amount of NADH consumed over a three-day trial run at room temperature was monitored daily by UV absorption of 340 nm indicated a consumption of 0.4 mM in 72 hours. NMR was conducted on the samples (Figure 12), which indicated the reactor had ceased after 2 days of operation. Evident from these data were that all the consumables (1 mM NADH and ATP) had been converted likely accelerated by thermal degradation. More importantly, however, were the presence of carbohydrate proton signals at 3.83 and 3.94 ppm, which have been assigned to protons within the carbohydrate rings of F6P, FBP, Xu5P, R5P, Ru5P and RuBP.

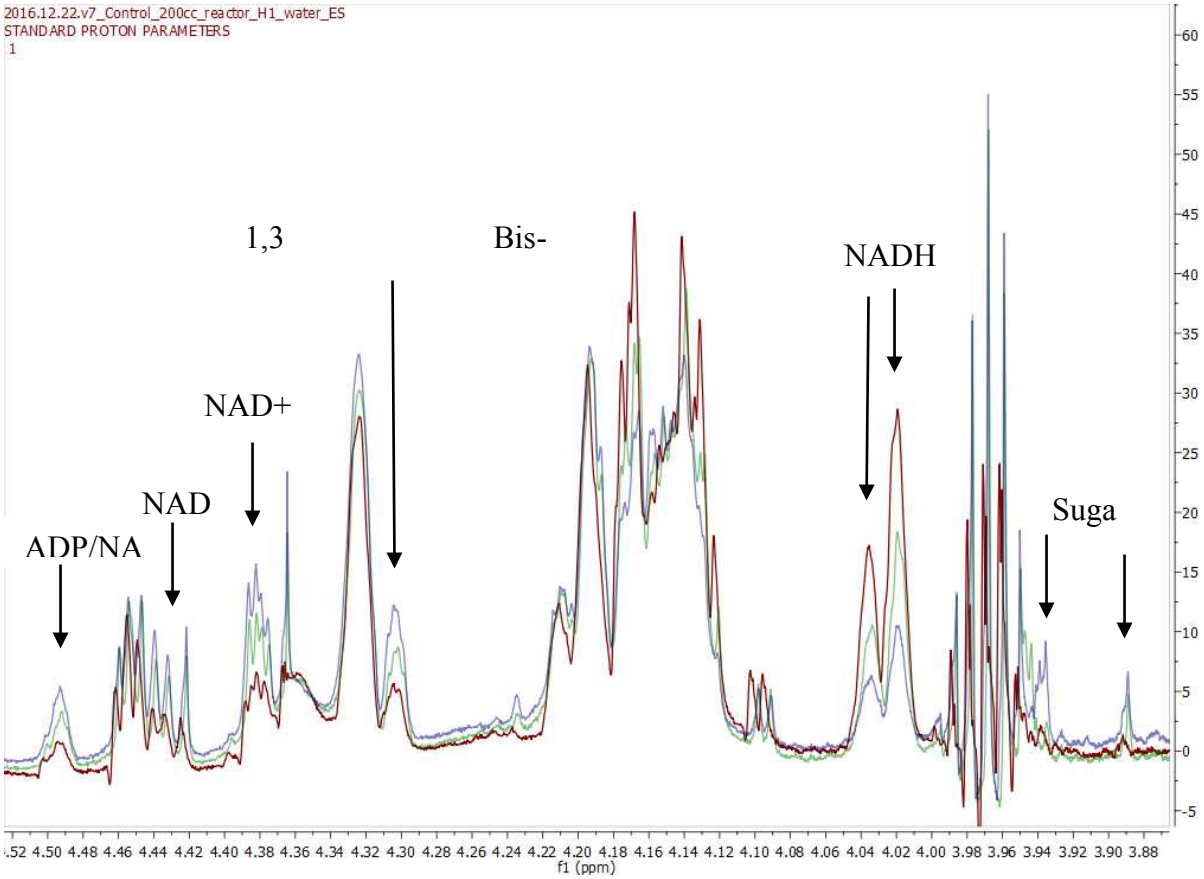

Figure 12- . Superimposed region of the 1H spectrum collected at 700 MHz for the prototype TFF immobilized Calvin cycle reactor at different time points: 24 hours (red), 48 hours (green) and 72 hours (blue). ADP chemical shift signal changes are shown by the loss of 4.32 ppm and the gain at 4.30 ppm. NADH chemical shift changes are evident from the loss at 4.02 and 4.04 ppm and gain of signals at 4.38 ppm. Most significant is the conversion of 3PG to BPG demonstrated by the downfield shift and build up of multiplet signal at 4.30 ppm from 4.21 ppm. Additionally, the build up of the other ATP dependent reactant Ru5P could result in the multiplet at 3.85 ppm. Furthermore, the multiplet at 3.94 ppm is unique to the sugars S7P, F6P, FBP, R5P, Ru5P and RuBP. Thus, demonstrating a functional cycle. Integration of these peaks quantifies the total sugars at about 100 uM, which is double the RuBP used to initiate the reaction (48 uM). Interestingly, it indicates the reaction slowed down considerably or ceased after 48 hours once the consumable metabolites NADH and ATP were spent, likely from thermal degradation as the 340 nm absorbance indicated a total reduction of NADH to be 400 uM in this time frame. This should have been enough to convert 200 uM of RuBP.

None the less, we indicated that the concept of the enzymatic cycle in free form could be achieved (Figure 9).

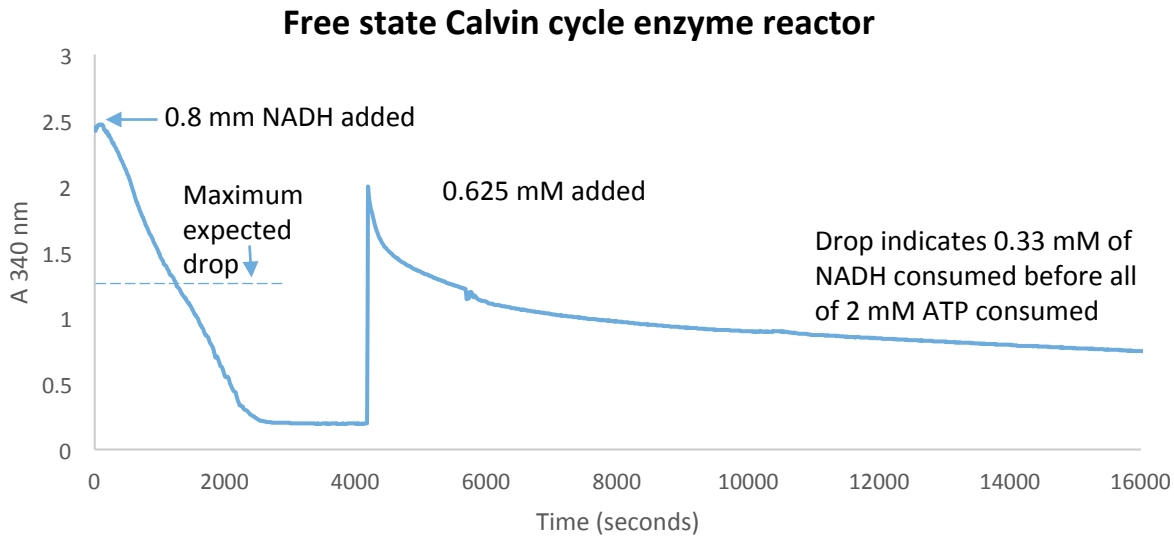

Figure 13- 340 nm trace o the full Calvin cycle enzyme reactor without NADH and ATP regeneration systems

Our data indicated that the cycle could be initiated over 3 days with the simple addition of consumables. No additional RuBP other than the 100 uM that was used to start the cycle was added on three consecutive days. The amount of NADH consumed was three times in excess of that compared with the RuBP used to start the cycle indicating that RuBP was in fact being generated and the cycle could be continued.

These enzyme ratios were used to build a complete closed cycle reactor with immobilized enzymes. The reactor was run for 3 consecutive days at 20 ºC. The NMR data indicates a consistent loss of bicarbonate CO_3 over this time period with an increase in the concentration of sugar. While the rate was less than expected, the limited sampling rate only provided an estimate of the real reactor turnover rate. Albeit slow, the proof of concept has been demonstrated that the ability to perform ongoing Calvin cycle consumption of CO_2 within a closed system reactor is feasible.

The results related to the immobilization showed that we should work on new strategies for immobilization of the enzymes since we are getting close to 10% efficiency with hollow fiber filter modules immobilization.

5.7 System Integration

The full cycle set-up included the enzymes of the Calving Cycle immobilized in hollow fiber modules. Figure 14 shows a schematic of the set-up. Some TFFs only contained one and some included multiple enzymes; their amounts are also shown. Two UV/Vis spectrometers were used to measure NADH concertation, before and after NADH regeneration as shown in Figure 14.

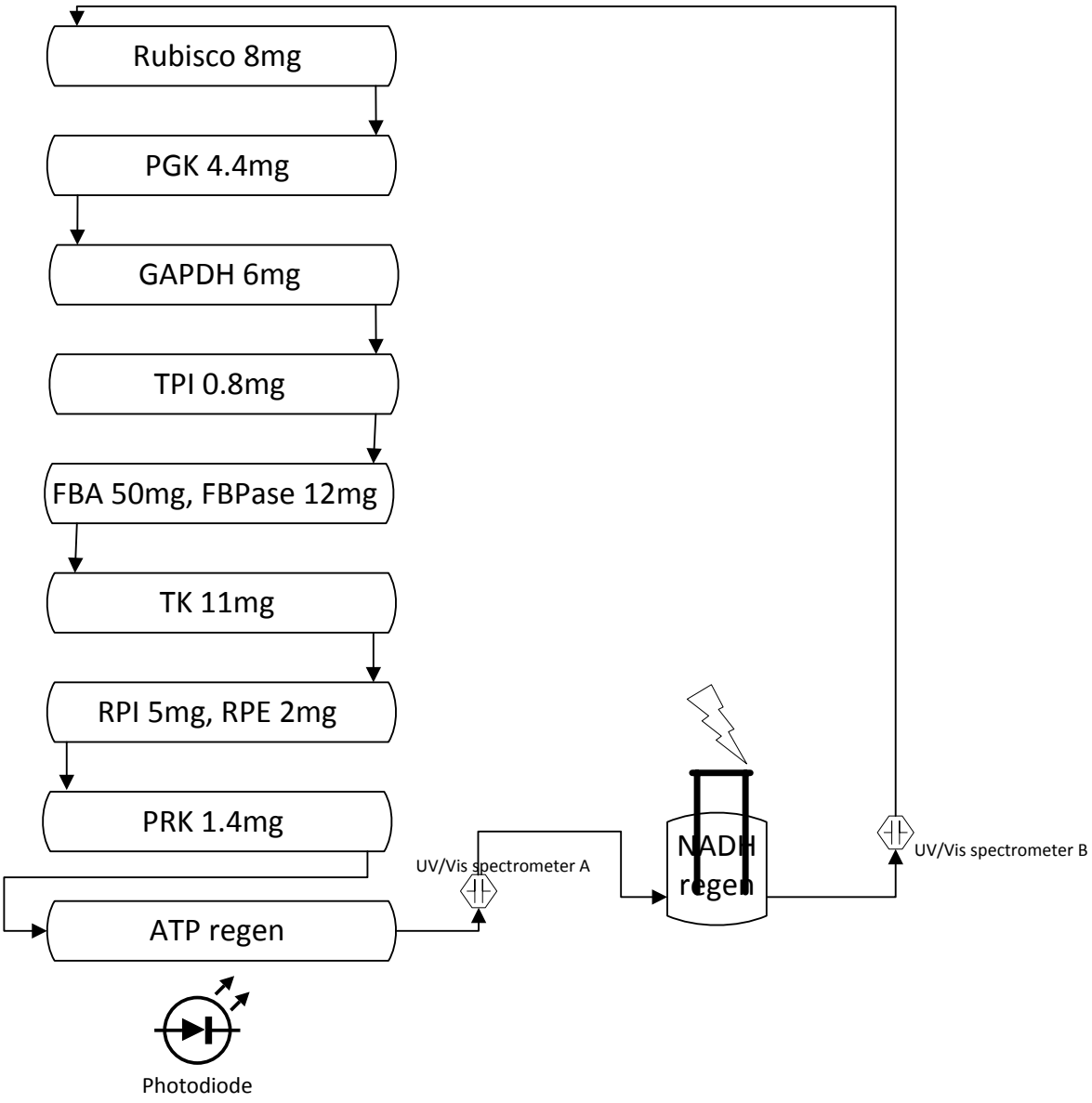

Figure 14- Schematic of the full Calvin cycle experiment

NADH has a signature absorption peak at 340nm. Therefore, absorption at 340nm correlates with NADH concentration, which is shown in Figure 15. The full cycle experiment was conducted over multiple days. NAD+ regeneration was accomplished on a glassy carbon electrode in an electrochemical cell. ATP was generated by vesicles that were trapped and recirculated in the final hollow fiber module.

The process fluids initially contained NAD+ and ADP. ADP was regenerated to ATP by shining LED light to the vesicles trapped in the last hollow fiber module. NAD+ was regenerated to NADH over two hours. Then NAD+ regeneration was stopped to allow the detection of its reduction over a one-day span. This would allow for confirming successful integration of NADH and ATP regeneration subsystems. Then regeneration was done again to replenish NADH concentration.

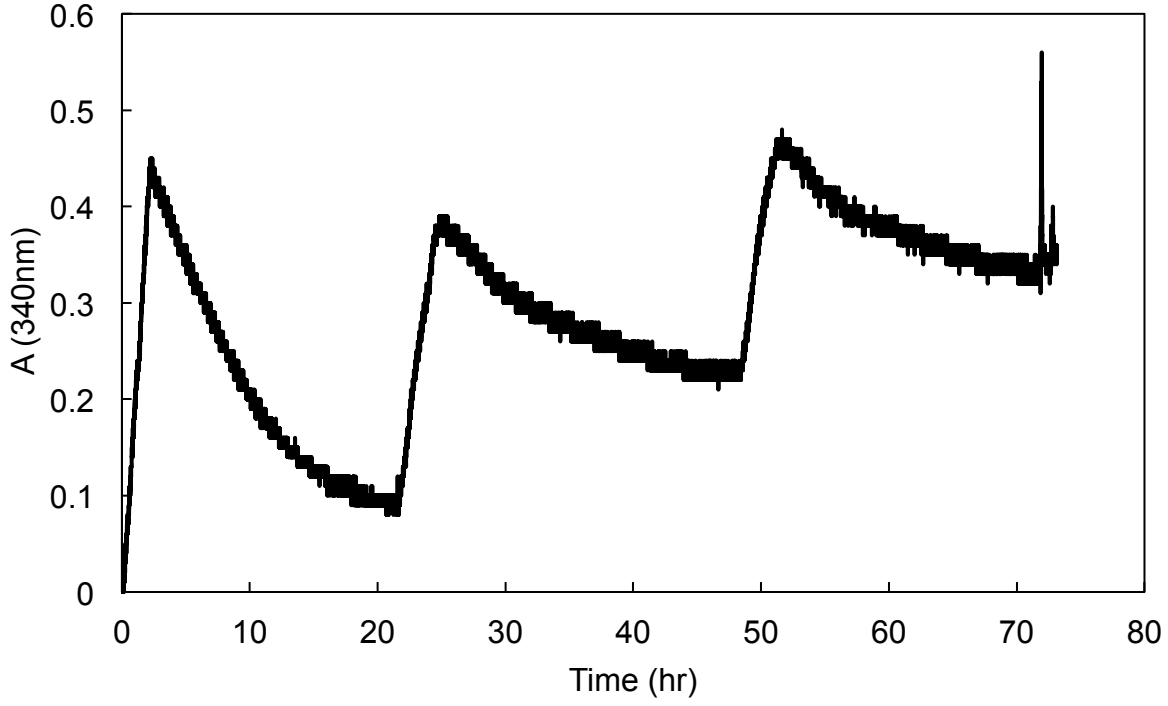

Figure 15- UV/Vis spectrometer trends at 340 nm which corresponds to NADH concentration.

Over the 3-day period, regeneration was done 3 times (3 cycles) which can be seen as sharp increases in Figure 15. The corresponding rates for NADH increase and decrease during the 3 cycles are shown in Table 4. The concentration of regenerated ATP in the integrated system was around 35 μM.

Table 4- NADH concentration increase and decrease rates.

Cycle (day)	1	2	3
Regeneratopn (μmole/min)	0.50	0.20	0.19
Disappearance ((μmole/min)	0.04	0.02	0.02

5.8 Greenhouse Gas Impacts

Life Cycle Assessment, as a tool to analyze the environmental impacts, is used to study the GHG emissions impact of our proposed process in case of ethylene glycol (EG) and dihydroxyacetone (DHA) production as potential final products, to compile the inventory of relevant energy and material inputs, environmental releases, and produced waste associated with enzyme production and bio-chemical processes. To achieve this goal, the current cradle to gate study covers the following two aspects:

1) Enzyme production: The process of producing immobilized enzymes for the process, and
2) Bio-chemical process: The process of producing EG and 1,3-dihydroxyacetone DHA from CO_2 as the feed using the enzymatic reaction platform.

Enzyme production comprises of different processes and sub-processes such as media preparation, fermentation, separation, cell disruption, immobilization, sanitization, and waste management [37]. Twelve enzymes in this study are produced and immobilized for use in the bio-chemical process. In the current investigation, enzyme production, mass balance, and energy flow are based on GSK standard operating procedure. Life cycle inventory (LCI) information was obtained from GSK in-house LCI database FLASC^{TM} [38], Ecoinvent [39], and the literature [40, 41].

In the proposed process, energy generation and water production are zero. There is a stream of produced non-hazardous waste which will be disposed based on the standard procedure [42]. There is no hazardous waste in the proposed process, so non-hazardous waste control regulations can be used for waste management in Alberta [43]. The enzymes pose no environmental hazards as they are quickly inactivated and biodegraded in the environment. Used enzyme in inoculation is not considered in the system boundary, because the use of it is negligible. Associated GHG emission regarding the produced waste has been considered in LCA analysis. Eutrophication and chemical/photochemical smog formation is assumed to be zero. Cradle to gate life cycle analyses of producing EG and DHA using conventional processes account for 0.763 kg CO_2/kg EG and 3.9 kg CO_2/kg DHA, respectively [44]. Therefore, our product reduces emissions by such amount through replacing those products in the market. The CO_2 emissions of our process would be minimized by use of photovoltaic cells to power our process. In that case, every kg of processed CO_2 will be considered a kg of captured CO_2. As it will be discussed later, this may not be the most economical scenario. Nevertheless, even if 100% grid power is used, accounting for the emissions associated with grid electricity, our calculations indicate that 0.07 kg of CO_2 is directly captured per kg CO_2 processed, which practically means zero emission for producing DHA and EG. Considering the 2.65 kg CO_2 indirect reduction per kg CO_2 processed (molar average of DHA and EG) our process significantly reduces CO2 emissions due to replacement of high CO_2 intensity chemicals. Comparing our proposed process with conventional chemical approaches highlights the big opportunity to save energy and to mitigate GHG emissions (Table 4).

Table 5- Green House Gas Impact

Performance Indicator	Target Value	Achieved by date
Five year GHG savings (post project completion)	1.44Mt/year*	2023
Ten year GHG savings (post project completion)	3.84 Mt/year*	2028

* Our technology is in the stage of lab-scale and these values are potential values for future commercial scale facility (1200 kg CO_2 per day) considering 3 and 8 % of capturing of total CO_2 emission from oil and gas sector in Canada after five and ten years of technology deployment, respectively.

6 Overall Conclusions

The compartments involved in this technology including ATP regeneration, NADH regeneration and production of Calvin Cycle enzymes were individually developed successfully. We could also integrate the system and run all the compartments. However, the methodology for immobilization of the enzymes needs to be modified since it was not efficient as expected.

We believe this technology can be a disruptive technology for the chemical industry and beyond that monumental for the humankind. While the conventional methods for producing chemicals has been heavily reliant on fossil based feedstock, our technology uses carbon in its lowest energy form and, harnessing the sunlight's energy, builds up small organic molecules, one molecule at a time. In essence, our process takes advantage of what nature has taught us in practice through millions of years - to take advantage of the resources that are readily available in contrast to using the sunlight's legacy energy in from of fossil resources. In a sense, nature is teaching us how to fish instead of presenting us the fish. Our process has almost unlimited potential for end-products, products that can be used as commodity chemicals, pharmaceutical, cosmetic, or even food ingredients. This presents an opportunity for providing the market with an ultra-green alternative of chemicals, chemicals that the minimum possible amount of energy and resources is used compared to any other process imaginable - even cell based biotechnology. In the ultimate fossil-resource free future, cars can run on electric power saved in batteries of some sort, and along with that., chemicals will be produce by utilizing energy to upgrade CO_2 into organic chemicals. In such a scenario, our process will be the most resource efficient, in terms of water and energy and the safest and least hazardous. However, our technology is in preliminary stage and we should continue to improve the efficiency of different involved compartments. We should work on increasing the production yield and turnover of our enzymes as well as finding an efficient way of immobilization methodology with minimum loss of activity. Also, we should work on engineering aspects of the technology in order to move to another stage.

7 Scientific Achievements

NRG COSIA Carbon XPRIZE semi-finalist project: http://carbon.xprize.org/teams

Montemagno, C. and Jenab, E. Artificial Photosynthesis as a Novel Management Tool for Industrial Carbon Dioxide Emissions. Innovative Solutions for a Sustainable World, Halifax, Nova-Scotia, Canada, July 3-6, 2016.

Mussone, P., Espinoza, M.I., Christensen, M., Rafie Borujeny,E., Minor, K., Nish, G., Wang, F., Semenchenko, V., Montemagno, C., "Artificial Photosynthesis as a Novel Management Tool for

Industrial Carbon Dioxide Emissions," BCN-AI Bio 2015 Conference, Edmonton, Alberta, November 22-25, 2015

Minor, K., Montemagno, C., "Artificial Photosynthesis Through Coupling Integral Membrane Proteins," 5th Annual Congress of Nano Science and Technology, Xi'an, China, September 24-26, 2015.

Minor, K., Montemagno, C.D., "Artificial Photosynthesis for Managing Industrially Produced CO_2 Emissions", BMMP-14, Japan, January 24-27, 2014.

8 Future Plan

Ingenuity Lab entered to NRG COSIA Carbon XPRIZE global competition and we became semi-finalist. The NRG COSIA Carbon XPRIZE is challenging the world to reimagine what we can do with CO_2 emissions by incentivizing the development of technologies that convert carbon from those emissions into valuable products. The second milestone of XPRIZE is to at least capture 60 kg of CO_2 per day and to convert it to valuable chemicals. We are currently working towards the goal assigned by the XPRIZE.

9 Communications Plan

Ingenuity Lab has commenced on an ambitious communications plan including an extensive social media presence as well as press releases, and traditional interviews. In order to communicate information about the project with third parties, Ingenuity Lab will be pursuing scientific papers as well as presenting results at scientific conferences and industry events. Due to our involvement with the NRG COSIA Carbon XPrize, we also have the opportunity to present our findings at XPrize specific events and will be featured on their website and social media channels. Ingenuity Lab is also featuring the results of this project on our website and social media channels to effectively engage the public to the results of this project. Ingenuity Lab is also currently producing a written supplement describing this project which will be packaged and distributed to the entire readership of The New Economy Magazine with their Spring Issue (May 2017). This supplement will also be distributed to key stakeholders of Ingenuity Lab and the digital copy will be featured on our social media streams and website, as well as The New Economy's website.

Below is a list of traditional press pick up for this project.

Date	Media Outlet	Title
April 28, 2015	Innovation Anthology	*Age of biological systems: Carbon capture and conversion*
May 24, 2016	Edmonton Journal	*Edmonton researchers aiming for X-Prize by making artificial plants*
May 25, 2016	Metro Edmonton	*Edmonton research group copies plants to cut carbon emissions*
July 15, 2016	The New Economy	*Ingenuity Lab looks to transform carbon into useful substances*

July 22, 2016	Eniday	*Climate Fighters*
October 19, 2016	Engadget	*Carbon XPrize chooses the 27 best solutions for CO2 emissions*
October 19, 2016	The New Economy	*Ingenuity Lab advances to the second stage of XPRIZE competition*
November 8, 2016	Yahoo! Finance	*Ingenuity Lab Carbon Solutions advances in $20M NRG COSIA Carbon XPRIZE*
November 8, 2016	JWN Energy	*Carbon CPRIZE teams seek to convert CO2 into products ranging from building materials to toothpaste*
November 8, 2016	Canadian Insider	*Ingenuity Lab Carbon Solutions Advances in $20M NRG COSIA Carbon CPRIZE*
November 8, 2016	Hydrocarbon Engineering	*Ingenuity Lab Carbon Solutions advances in NRG COSIA Carbon XPRIZE*
November 8, 2016	Carbon XPrize	*27 Teams advancing in NRG COSIA Carbon XPrize*
November 8, 2016	Canadian GreenTech	*Nine Canadian companies vying for top Carbon XPrize after reaching semifinals*
November 8, 2016	Market Wired	*Ingenuity Lab Carbon Solutions Advances in n$20M NRG COSIA Carbon Xprize*
January 9, 2017	CBC News	*9 Canadian teams reach semifinals of $20M Carbon XPrize competition*
January 9, 2017	Alaska Highway News	*Canadian teams reimagine carbon dioxide emissions for $20M competition*
January 10, 2017	The Edmonton Journal	*Canadian teams reimagine carbon dioxide emissions for $20M competition*
January 10, 2017	Les Devoir	*Un concours pour trouver la meilleure technologie verte*
January 10, 2017	Edmonton Metro	*Edmonton Lab makes the finals for global Carbon XPrize*
January 11, 2017	National Observer	*Canadian teams reimagine carbon dioxide emissions for $20M competition*
January 11, 2017	Vancouver Observer	*Canadian teams reimagine carbon dioxide emissions for $20M competition*
January 11, 2017	CBC News	*Edmonton entry in the Carbon Xprize*
January 12, 2017	The Ryan Jesperson Show	*Reimagining carbon dioxide*
January 12, 2017	NewsTalk 610 CKTB	*The VIP late lunch with Lee Sterry*

10 Final Financial Report

Final financial report is provided in a separate document.

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