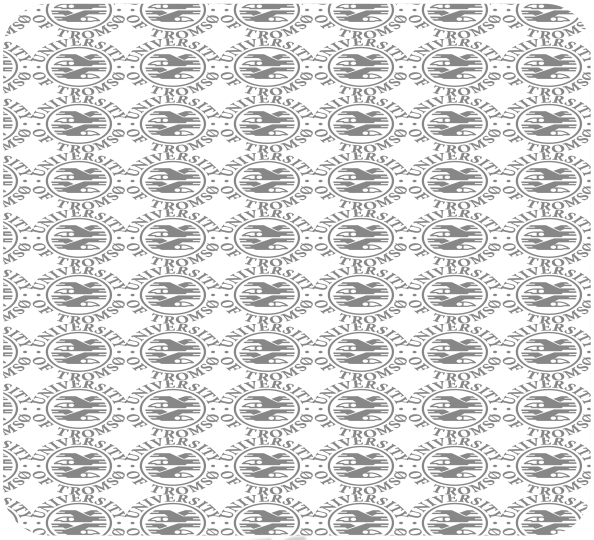
Master thesis in Medical Biology

Cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes



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Thanks for keeping up with me in all this time. Be blessed always and have a bright future.

Thank to all those who helped me in any ways to be here today.

Summary

The transport of cyclic nucleotides out of the cell is energy requiring, dependent on ATP-hydrolysis. The ability to stimulate this ATPase is a hallmark of substrates for ABC-transporters e.g. ABCC5, ABCC4. It is believed that ABCC5 transporter has selective high affinity for cGMP. Previous studies support the idea that ABCC5 contribute to cGMP transport by human erythrocytes. Human erythrocyte membrane possesses cGMP transport system that utilizes ATP for its activity. Present study was conducted to measure, "Cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes" by an In-house assay method and to compare it with commercially available kit to make cost benefit analysis.

The main findings were: 1) The inorganic phosphate standard curves were linear for relevant biological concentrations, observed for both in-house and commercial assay. 2) The membrane protein (i.e. IOV) concentrations raised the inorganic phosphate concentrations linearly. 3) It was possible to distinguish cGMP-stimulated activity from basal ATPase activity in hRBC IOV, even though the difference was small. 4) The two phosphate assays (in-house and commercial) had both advantages and disadvantages, none being superior to the other one.

Table of Content

Abbreviations:

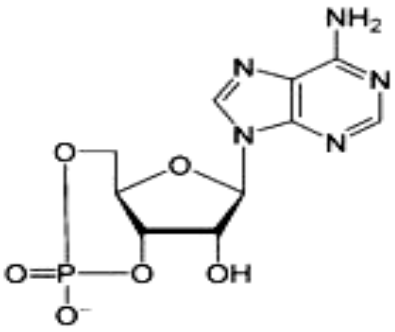
Abbreviation	Meaning
ABC-transporters	ATP-Binding –cassette transporters
ABCC4	Multidrug resistance-associated protein 4
ABCC5	Multidrug resistance-associated protein 5
Abs.	Absorbance.
AchE	Acetylcholinesterase.
AC	Adenyl cyclase.
ADP	Adenosine diphosphate.
AMP	Adenosine monophosphate
ANP	Atrial natriuretic peptide.
ATCC	Acetylthiocholine-chloride.
ATP	Adenosine triphospahte.
ATPase	Adenosine triphosphatase
BC	Reagent B
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
Conc.	Concentrations
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Exp	Experiment
FIQ1	Final incubation #1
FIQ2	Final Incubate # 2
GAPD-activity	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein coupled receptors
GTP	Guanylyltri phosphate
hRBC	Human red blood cells
IOV	Inside out vesicles
IQ1	Incubation solution #1 with cGMP

IQ2	Incubation solution # 2 without cGMP
IS1	Incubation solution S1
IV0	Inside out vesicles with 0 detergent
IVX	Inside out vesicles with X-100.
Mg-ATP	ATP.magnesium salt
$MgCl_2$	Magnesium chloride
MRP4	Multidrug resistance-associated protein 4 = ABCC4
MRP5	Multidrug resistance-associated protein 5 = ABCC5
NO	Nitric oxide
OD	Optical Density.
PKA	Protein Kinase A
PDE	Phosphodiesterase.
PKG	cGMP dependent protein kinase
PGC	Membrane bound guanylyl cyclase.
pH	Hydrogen ion concentration.
Pi	Ortho phosphate.
RBC	Red blood c ells
ROV	Right side out vesicles.
SDS	Sodium dodycyl sulphate
SGC	Soluble guanylyl cyclase
Sr.#	Serial number.
Sted.Dev.	Standerd deviation

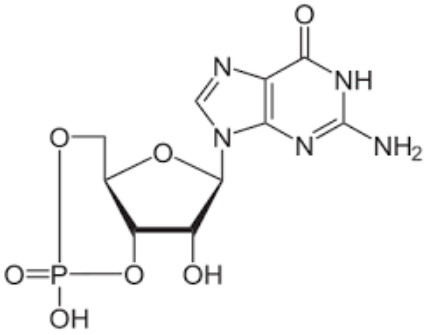
1. INTRODUCTION

1.1. Cyclic nucleotides

Cyclic nucleotides such as cAMP and cGMP are substances with a specific cyclic bond arrangement between the sugar and phosphate groups, and function as basic components in a communication system that acts within cells. They are important intracellular signal transduction molecules known as second messengers. [9]



Cyclic adenosine monophosphate (cAMP) [30]



Cyclic guanosine monophosphate (cGMP) [35]

Figure 1: Structures of cAMP and cGMP.

Inside the cell, second messengers transmit and amplify signals from receptors to downstream target molecules. They are rapidly synthesized and eliminated by cellular enzymes and by membrane transporters, rapidly sequestered in a membrane-bound organelle or vesicle or have a restricted distribution within the cell. [34]

The cellular biokinetics of cyclic nucleotides comprises three processes: synthesis, biotransformation and cellular extrusion. Precursors of cyclic nucleotides synthesis are ATP and GTP.

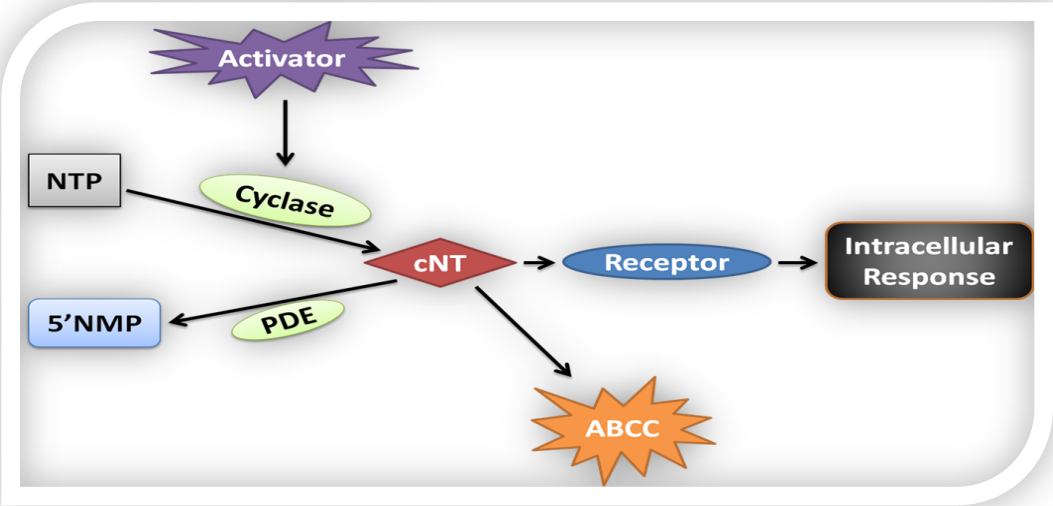


Figure 2: Cyclic nucleotides signaling pathway: NTP; nucleotide triphosphate, 5'NMP; 5'nucleotide mono phosphate, cNT; cyclic nucleotides, PDE; phosphodiesterase, ABCC; ABC- transporter. [9]

Cyclic AMP is produced in cells through the activity of membrane bound adenyl cyclase. [15] The majority of ACs are indirectly activated by various stimuli including adrenergic agonists, which bind to G protein coupled receptors (GPCRs) on the cell membrane resulting in the activation of the GPCR and release of the $G\alpha_s$ subunit that is subsequently responsible for binding to and activating AC, thus stimulating the production of cAMP.[11, 17] Cyclic AMP is biotransformed into AMP by members of the phosphodiesterase enzyme family [31] and extruded unmodified to the extracellular space by ABCC transporters.

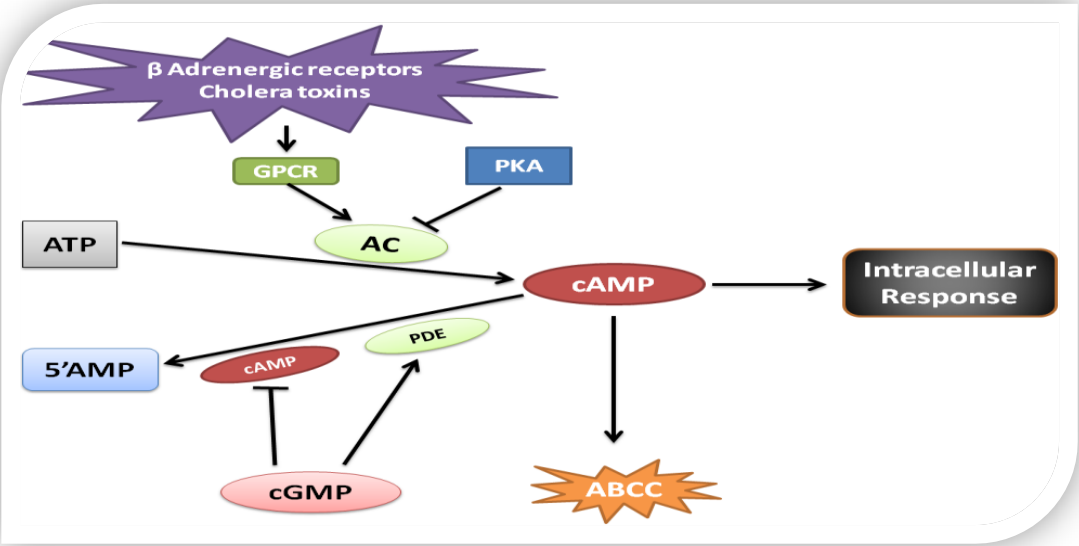


Figure 3: cAMP signaling pathway: ATP; Adenosine tri phosphate, 5'AMP; 5' adenosine mono phosphate, GPCR; G protein coupled receptor, AC; adenyl cyclase, PKA: Protein kinase A, CAMP; cyclic adenosine mono phosphate, cGMP; cyclic guanosine mono phosphate PDE; phosphodiesterase, ABCC; ABC- transporter. [9]

Due to the activity of members from the guanylyl cyclases GTP is converted to cGMP. [34, 31, 9] There are two different classes of guanylyl cyclases, the membrane bound form (PGC) and the soluble form (SGC). The PGC and SGC are stimulated by atrial natriuretic peptide (ANP) and nitric oxide (NO) respectively. Activity of phosphodiesterases decomposes cGMP in to GMP which is extruded unchanged by ABCC transporters. [31]

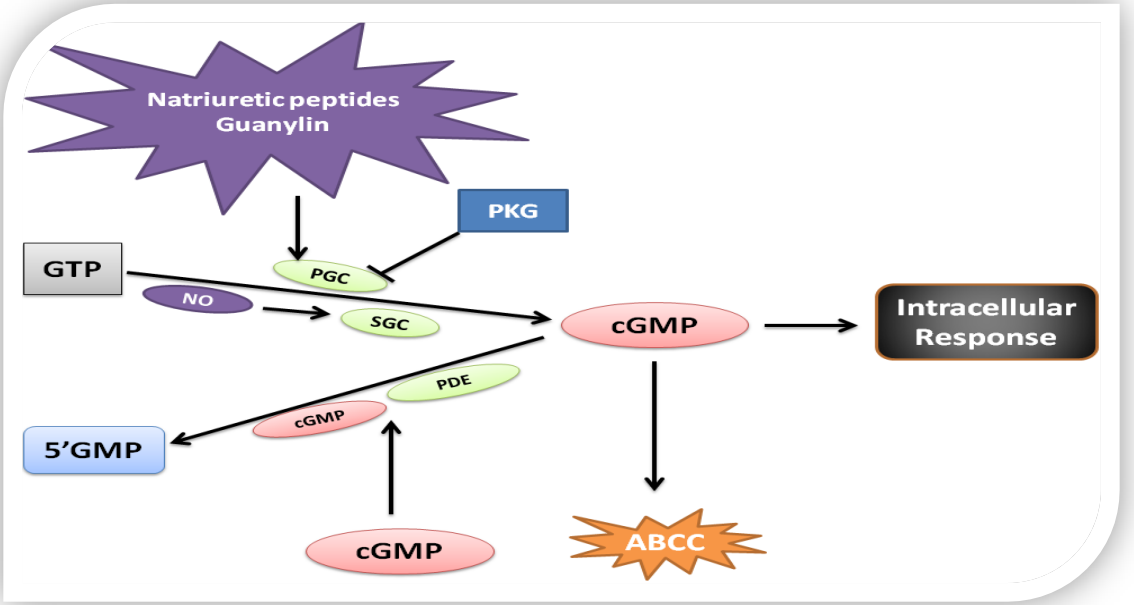


Figure 4: cGMP signaling pathway: GTP; Guanosine tri phosphate, 5'GMP; 5' guanosine mono phosphate, PKG; cGMP dependent protein kinase, PGC; membrane bound guanylyl cyclase ,SGC; soluble guanylyl cyclase, NO; Nitric oxide, cGMP; cyclic guanosine mono phosphate, PDE; phosphodiesterase, ABCC; ABC-transporter [9]

While the specific function of a given signal varies according to

a) The cell type
b) Extracellular environment
c) Stimulus activating the signal
d) Localization of the signal and
e) The type of cyclic nucleotide formed.

Both the amplitude and duration of a cyclic nucleotide signal also vary and are largely dependent on the expression and activity levels of cyclic nucleotide phosphodiesterase (PDE) enzymes, which is responsible for hydrolyzing the cyclic nucleotide in order to terminate the signal. [9,18] It is commonly accepted that the total concentrations of cAMP and cGMP in

most cells are typically from below 1 µM up to 10 µM but the active free concentration being far lower. The transport of cyclic nucleotides out of the cells is energy requiring, dependent on ATP- hydrolysis. [29]

1.2. ATPases

ATPases represent a large enzyme family by which some members can store chemical energy into high-energy phosphoanhydridic bonds in ATP and other members can utilize the stored energy for mechanical work such as transport of small inorganic or organic molecules against huge concentration gradients. An example of the first class is the F-type (F_o F_i type) or ATP synthase/H^+ ATPase complex and examples of the second class is P-ATPases (transport ATPases) and m-ATPases (ABC-transporters). [19] The ability to stimulate ATPase is a hallmark of substrate translocation by ABC-transporters. The end products of the ATPase activity are ADP and an inorganic phosphate, orthophosphate (Pi). The enzyme activity can be determined by measuring the release of inorganic phosphate by colorimetry

$$ATP \xrightarrow{\text{ATPase activity}} ADP + P_i$$

1.3. ATP Binding Cassette (ABC) Transporters

ABC-transporters belong to one of two principal transporter super-families, affecting cell membrane translocation of drug. ABC-transporters are primary active transporters requiring ATP hydrolysis. The mechanism includes the following steps,

1. Opening of the channel toward the inside of the cell.
2. Substrate binding and conformational changes in the ATP-binding cassettes.
3. ATP binding and further conformational changes.
4. Separation of the membrane-binding domains and release of the substrate to the other side of the membrane.
5. ATP hydrolysis to reset the transporter to its initial confirmation.

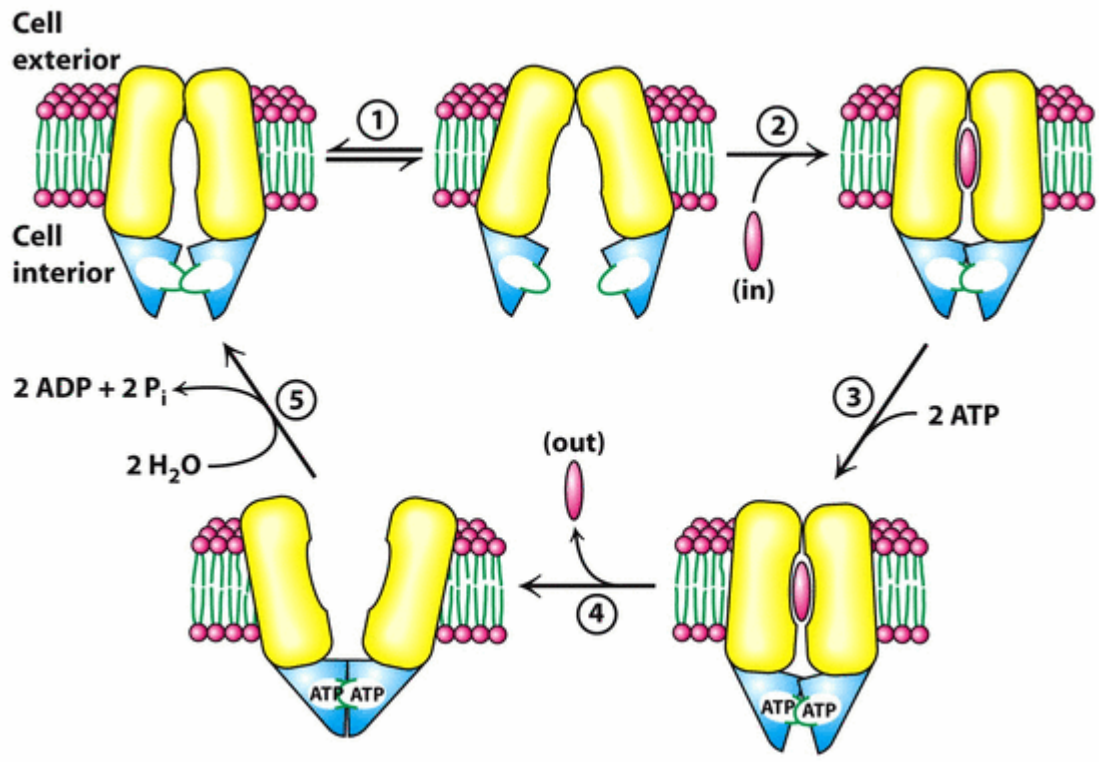


Figure 5: ATP Binding Cassette Transporter mechanism [32]

Members of subfamily C among the ABC-transporters play an important role in the extrusion of cyclic nucleotides. ABCC4 (MRP4) and ABCC5 (MRP5) are most important for the cellular extrusion of cGMP and cAMP. Previous studies of ATP-dependent transport support the idea that ABCC5 has high affinity for cGMP while ABCC4 has high affinity for cAMP. [3] ABCC5 contributes to cGMP export from human erythrocytes. At higher concentrations ABCC4 and ABCC5 can transport both cyclic nucleotides. [3]

1.4. Inside out vesicles from human erythrocytes.

The different intracellular and extracellular behavior of biological membrane is result of the membrane organization. To characterize individual surfaces, one possible technique is to seal the membrane in the form of vesicles. These vesicles could be of the types,

- Inside out vesicles (IOV): Cytoplasmic side of the membrane is outward.
- Right side out vesicles (ROV): The external surface is still outward.

For pharmacological studies of cyclic nucleotide extrusion, hRBC which possesses ABCC4 [16, 20] and ABCC5 [14, 16, 7, 4] are suitable choice. [33] These cells are easily and freshly obtainable and preparation of IOV makes it possible to characterize ATPase activity after stimulation with cGMP.

1.5. Aims of the work

In the present work we have characterized an assay for determination of inorganic phosphate concentrations and employed this method to study basal and cGMP-stimulated ATPase activity in IOV from hRBC.

The work aimed to

1) Test the linearity of the inorganic phosphate standard curve.

2) Determine the influence of membrane protein concentrations on the linearity of the inorganic phosphate assay (dilution studies).

3) The ability to distinguish basal ATPase activity from cGMP-stimulated activity in hRBC IOV and finally,

4) Compare the in-house method with a commercially ATPase available assay kit (Innova Biosciences) to make a cost benefit analysis.

2. Material and methods

2.1. List of chemicals

An overview of chemicals used is given below. Detailed in formation of the chemical materials that were used to perform the experiments in the present work is shown in Appendix7.1.

Substance (chemical formula)	**Supplier**	**Code**
Sodium chloride (NaCl)	Sigma-Aldrich	S 5886
Potassium chloride (KCl)	Sigma-Aldrich	P 31248
Potassium hydroxide (KOH)	Merck	B 675233
EGTA ($(CH_2OCH_2CH_2N(CH_2CO_2H_2)_2$)	Sigma-Aldrich	E 3889
HEPES ($HOCH_2CH_2$-N(-C_2H_2-)$_2$N-$CH_2CH_2SO_3H$)	Sigma-Aldrich	H 4034
Magnesium chloride ($MgCl_2$•$6H_2O$)	Merck	5833
Pyrophosphate ($Na_4P_2O_7$•$10H_2O$)	Fluka	71514
Phosphate basic (Na_2HPO_4•$2H_2O$)	Sigma-Aldrich	S 6751
Sodium hydroxide (base) NaOH	Merck	B 0312669
Protease inhibitor cocktail	Sigma	P 8340
Cysteine ($C_3H_7NO_2S$)	Fluka	30090
β-Nicotinamide adenine dinucleotide (NAD)	Sigma-Aldrich	N 3014
5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)	Sigma- Aldrich	D 21820
Histodenz	Sigma	D 2158
Tris $(HOCH_2)_3CNH_2$	Merck	1.08382
Tris concentrated, acidic. (Tris•HCl)	Merck	1.08219
Potassium phosphate, acidic ($KH_2PO_{4)}$	Merck	1.04873
Potassium phosphate basic (K_2HPO_4•$3H_2O$)	Sigma	P 5629
Sodium dodecyl sulphate	Sigma-Aldrich	L 5750
Ouabain g-strophanthin ($C_{29}H_{44}O_{12}$)	Sigma- Aldrich	O 3125

Ammonium molybdate $(NH_4)_2MoO_4$	Sigma	A 7302
Ascorbic acid ($C_6H_8O_6$)	Sigma-Aldrich	A 5960
Hydrochloric acid (HCl 37 %)	Sigma	A 30721
ATP (Na_2ATP)	Sigma	A 2383
Arsenate ($Na_2HAsO_4 \bullet 7H_2O$)	Fluka	71625
Acetylthiocholine-chloride (ATCCs)	Sigma	A5626
Triton X-100	Sigma	T 8787
Cyclic guanosine monophosphate (cGMP)	Sigma	G 6129

2.2. Buffers and Solutions

An overview of the buffer and solutions used is given below. Detailed information of the buffers and solutions is given in Appendix 7.2, 7.3 and 7.4.

Buffer for cell washing

HEPES buffer (HEPK): 10 mM, K^+ 8.6 mM , EGTA 2 mM, NaCl 150 mM, pH = 7.50

Phosphate Buffered Saline (PBS): Phosphate 10 mM, NaCl 137 mM, KCl 2.7 mM, pH = 7.40

Buffers for cell lysis

Lysis buffer # 1 (L1): EGTA 0.5 mM, $MgCl_2$ 2.0 mM, NA*2ATP 0.6 mM, HEPES 20 mM, K^+ 28.9 mM, pH = 7.50

Lysis buffer # 2 (L2): EGTA 0.5 mM, HEPES 20 mM, K^+ 28.9 mM, pH = 7.50

Buffers for IOV preparation

Hypotonic lysis buffer A: Tris 5.0mM, K^+ 113 mM, Cl^- 116mM, pH = 8.1

Hypotonic washing buffer B: Tris 5.0mM, EGTA 0.5 mM, K^+ 4.0 mM, Cl^- 5.6 mM, pH = 8,1

Hypotonic vesiculation buffer C: Tris 0.5 mM, Cl^- 0.2 mM, pH = 8.2

Buffers for Sidedness

Phosphate buffer with Triton X-100 (FX): Phosphate 5.0mM, Triton X-100 0.2%, pH = 8.0

Phosphate buffer with 0 detergent (F0): Phosphate 5.0mM, pH = 8.0

Solutions for Sidedness test

Incubation solution for sidedness 2 (IS2): DTNB (5, 5' –dithiobis-(2-nitrobenzoic acid)) 0.7mM, phosphate 100mM, pH = 7.5

ATCC Solution: ATCC (Acetylthiocholine-chloride + water) 12.5mM. Store at -70° C.

IV0 (IOVs with 0 detergents): IOV + F0

IVX (IOVs with Triton X-100). IOV + FX

Solutions for ATPase assay for inside out vesicles:

Incubation solution # 0 (IQ0) - for determination of basal (unstimulated) level:

Tris 51.7 mM, EGTA 5.18 mM, ATP 10.35 mM, Mg^{2+} 10.35 mM, Ouabain 5.2 mM, K^{+} 728.6 mM, Cl^{-} 39.8 mM.(See safety aspects note 1in appendix 7.4)

Incubation solution # 1 (IQ1) - for determination of cGMP-stimulated level:

Tris 50.0 mM, EGTA 5.0 mM, ATP 10.0 mM, Mg^{2+}10.0 mM, Ouabain 5.0 mM, K^{+} 704.3 mM, Cl^{-} 715.1 mM, cGMP 50 µM (microMolar)

Incubation solution # 2 (IQ2) - for determination of without cGMP-stimulated level:

Tris 50.0 mM, EGTA 5.0 mM, ATP 10.0 mM, Mg^{2+}10.0 mM, Ouabain 5.0 mM, K^{+} 704.3 mM, Cl^{-} 715.1 mM, cGMP 0.0 µM (microMolar)

Final Incubate # 1 (FIQ1) - for determination of ATPase activity of IOVs with cGMP stimulation:

Tris 10.0 mM, EGTA 1.0 mM, ATP 2.0 mM, Mg^{2+} 2.0 mM, Ouabain 1.0 mM, K^{+} 140.9 mM, Cl^{-} 143.0 mM, cGMP 10 µM (microMolar), pH = 8

Final Incubate # 2 (FIQ2) - for determination of ATPase activity of IOVs without cGMP stimulation:

Tris 10.0 mM, EGTA 1.0 mM, ATP 2.0 mM, Mg^{2+} 2.0 mM, Ouabain 1.0 mM, K^{+} 140.9 mM, Cl^{-} 143.0 mM, cGMP 0.0 µM (microMolar), pH = 8

Reaction Inhibitor (SDS): Sodium dodecyl sulphate 12%

Coloring Reagent (BC): Ascorbate 3%, HCl 700mM, Ammonium molybdate 0.5 %.(See safety aspects note 2, 3 and 4 in appendix 7.4)

2.3. List of Individual procedures

1. Preparation of IOV from human erythrocytes (Red blood cells, or RBC).
2. Phosphate assay and different phosphate concentrations.
3. Phosphate assay and the effect of different concentrations of cGMP
4. ATPase assay for IOV.
    a. Phosphate assay of IOV in presence and absence of cGMP.
    b. Effect of different IOV concentrations on ATPase assay.
    c. Difference in absorbance in presence and absence of IOV.
    d. ATPase assay with higher IOV concentrations.
    e. ATPase assay with higher IOV concentrations with modified protocol.
    f. ATPase assay of IOV with commercial kit.

2.4. Preparation of IOV from human erythrocytes (hRBC)

Mature human erythrocytes have a maximal diameter of about 7.4 µm. Due to their extreme flexibility they can deform and pass through capillaries with a size of 1.5 µm. There are at least two reasons for this flexibility. First, during maturation the nucleus is expelled and the cells go into a final life span of 120 days in average. Secondly, the cell membrane has a specialized composition that favors flexibility. This is also the reason why the membrane of these cells under certain conditions invaginates to form IOV or protrude to form ROV.

In the present study, IOV were prepared with a modification of the method originally described by Steck. [24] Figure 6 shows the principle steps of the method. Fresh human EDTA blood was collected and all steps were performed at 0°C - 4°C. The cells were sedimented by centrifugation. RBC were washed three times with buffer A (pH 8.1) for hypotonic lysis. The cells were resedimented and lysed in 10 volumes of buffer B (pH 8.1), then rewashed in the same buffer B until ghosts were milky white. To initiate hypotonic vesiculation 49 volumes of cation free buffer C (pH 8.2) were suspended to one volume of cell suspension. The volume suspension is resedimented and homogenized by passing through a 27G cannula. A density gradient was used to separate IOV, ROV and ghosts by overnight ultracentrifugation. The uppermost band was collected, washed and resuspended in buffer C. A detailed procedure is described in appendix 7.5.1.

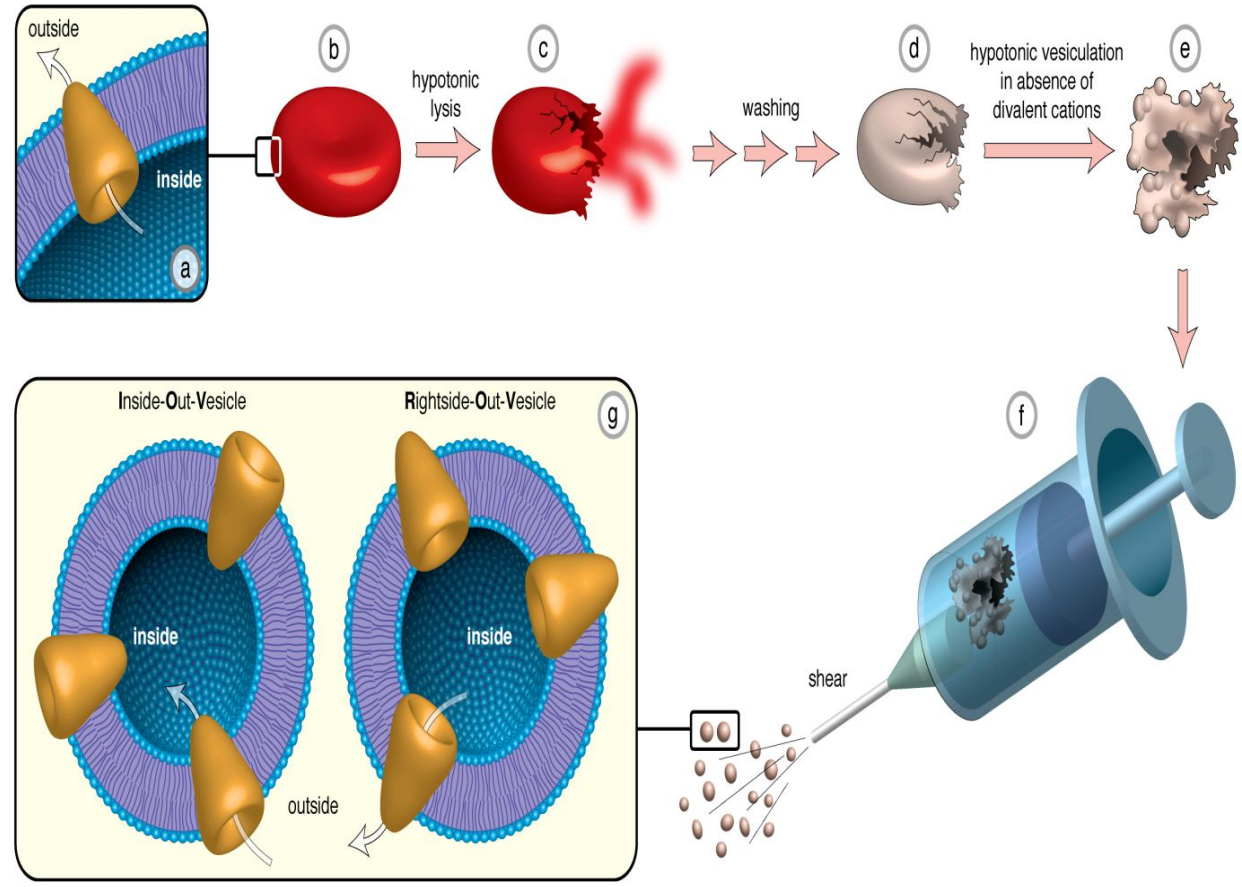


Figure 6: Diagramic illustrations of preparation of IOVs form hRBC (ill.: Roy Lysaa).

A: Enlarged view of normal human erythrocytes membrane. **B:** Human erythrocyte. **C:** Hypotonic lysis for the removal of cell contents. **D:** Erythrocytes ghosts after hypotonic washing. **E:** Hypotonic vesiculation after incubation with very low ionic strength alkaline buffer. **F:** Shear applied for homogenized sealed IOV. **G:** Enlarged view of an IOV and ROV.

2.5. Sidedness of vesicles

Using acetylcholinesterase accessibility sidedness of the ghosts was verified with small modifications to the original method. [8]

The enzyme acetylcholinesterase (AChE) is anchored to the cell membrane and is located at the extracellular side of the membrane. For the inside out vesicles the situation is the opposite. IOV should give low AChE-activity, while ROV should give high AChE-activity. By measuring the activity of this enzyme at the extravesicular side, the relative fractions of IOV and ROV can be found. The existence of AChE-activity in IOV is controlled by using the detergent Triton-X100 that makes the membrane leaky. In the presence of this detergent, any

extravesicular substrate will also become available for intravesicular enzyme; then both IOV and ROV will give high AChE-activity.

2.6. Phosphate assay and different phosphate concentrations

In 1925 Fiske and Subbarow first described the method of colorimetric determination of inorganic phosphate with ammonium molybdate complexes.[10] Since then, many modifications have been made to avoid organic phosphate hydrolysis during color development. [1, 2] .

Different concentrations of phosphates (5 µM, 15 µM, 25 µM, 50 µM, 100 µM and 250 µM) were prepared. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphates.

2.7. In-house phosphate assay

The ATPase activity is determined by measuring the release of inorganic phosphate generated from ATP hydrolysis by colorimetry.

To measure ATPase activity analytes are incubated in a solution or reaction medium with pH 8.0 that optimizes transport activity for the assay. The solution is incubated at 37℃ for suggested time and then reaction was stopped by using SDS 12%. Addition of coloring reagent is making a color complex with free phosphates. Absorbance of analyte is directly proportional to the amount of available inorganic phosphates

The ATPase activity of the ABC-transporter in hRBC IOV was determined by colorimetric method involving measuring the release of inorganic phosphates (P_i) during ATP-hydrolysis by Chifflet. [6]

While describing, “A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein” he used sodium dodycyl sulfate (SDS) to stop the enzymatic reaction resulting not to develop color immediately while working with large number of samples since there is no further organic phosphate hydrolysis. For color development, he used a freshly prepared solution of 6% ascorbic acid in 1 N HCl and 1 % ammonium molybdate. [6]

For current study to characterize ATPase activity in IOV of human erythrocytes after stimulation with cGMP, we used a modified version of method adopted from Chifflet and Boadu & Sager. [6] [3]

Inside out vesicles of human erythrocytes were stored at -70 C in microcentrifuge tubes. Required numbers of tubes was taken out of freezer and were kept in ice box at 4˚C. The thawn IOV were added in the presence of cGMP in a solution with pH 8.0 at 37˚C that optimizes transport activity are incubated for suggested time. The transport reaction starts when IOV are mixed in. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphates.

2.8. Commercial phosphate assay

Since 1925, different types of procedure have been used for the colorimetric determination of inorganic phosphate. Basic principle behind is, the formation of a colored complex of available P_i and coloring reagent used. Colorimetric detection of the color complex of ammonium molybdate and free P_i was a widely used technique for inorganic phosphates.

In 1965 Itaya and Koich, tested other dyes including safranin, brilliant green, malachite green, fuchsin red, methylene blue, methyl violet while working for "New micro method for the colorimetric determination of inorganic phosphate". They observed by adding 1 µg per ml of inorganic phosphate into the acidified solution of basic dyes containing ammonium molybdate resulted that malachite green not only produced the high intensity of color but also with a marked shift to the absorption maximum by exposure to phosphomolybdate, showed most significant behavior as the color developing reagent for micro-determination of phosphate. [13]

Commercial ATPase assay kit by Innova Biosciences:

The ATPase colorimetric assay kit by Innova Biosciences employs all reagents necessary for measuring ATPase activity. The kit contains P_iColorLock™ Gold reagent (an improved malachite green formulation) with additives to prevent background signals arising out of non-enzymatic ATP hydrolysis. Assay can be read anywhere in the wavelength range 590- 660nm.

Principle of the ATPase assay kit:

$$\underset{\text{ATPase}}{ATP \rightarrow ADP} + \underbrace{P_i \quad Dye}_{\substack{P_i\text{-dye Complex} \\ \textbf{(Green)}}}$$

3. Results

In order to measure "Cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes", a series of experiments were performed following the protocols. The raw data obtained is mentioned in Appendix 7.6. Final results, obtained are described in this chapter.

Firstly, inside out vesicles from human erythrocytes were prepared and tested for sidedness.

3.1. Sidedness test of IOV:

Three different batches of IOV were prepared and tested for sidedness, following yields were obtained.

Table 1: The table is about sidedness of vesicles and shows mean % yield for IOV and ROV obtained from three experiments.

	Batch 1	**Batch 2**	**Batch 3**
% IOV	50	26	28
% ROV	50	74	72

Total obtained volume of vesicles were distributed in 1mL aliquots, labeled them with percentage, date of preparation and stored at -70℃ to reuse them as per required.

For performing the next series of experiments to measure, cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes **,** the batch (batch 1) of vesicles produced with high yield of IOV (= 50%) was used to get maximum effective results.

3.2. Phosphate assay and different phosphate concentrations

Table 2: The table shows absorbance (at 680 nm) of different concentrations of phosphates in µM with and without cGMP. To measure absorbance for each phosphate concentration e.g. (5 µM) three parallels with cGMP and three parallels for same phosphate concentration but without cGMP were prepared to avoid possible errors. Absorbance measured at wavelength 680 nm for each parallel is represented by a, b, and c, also calculated the mean and standard deviation for both (phosphate concentration with and without cGMP).

Phosphate Conc.	Absorbance x 100									
	with cGMP					without cGMP				
µM	A	b	c	Mean	SD	a	b	C	Mean	SD
5	0,9	0,3	0,6	0,6	0,14	0,2	0,5	0,4	0,4	0,12
15	1,2	1,3	1,1	1,2	0,08	1,3	0,7	0,6	0,9	0,31
25	4,9	4,8	4,8	4,8	0,02	4,6	4,5	4,3	4,5	0,12
50	10	9,3	9,8	9,7	0,22	9,7	8,9	7,5	8,7	0,91
100	21,7	------	25,8	23,8	1,03	20,4	20	20,8	20,4	0,33
250	30,3	33,3	28,0	30,5	2,16	28,7	28,6	27,7	28,3	0,45

Figure 7: The figure shows mean values from table 2.

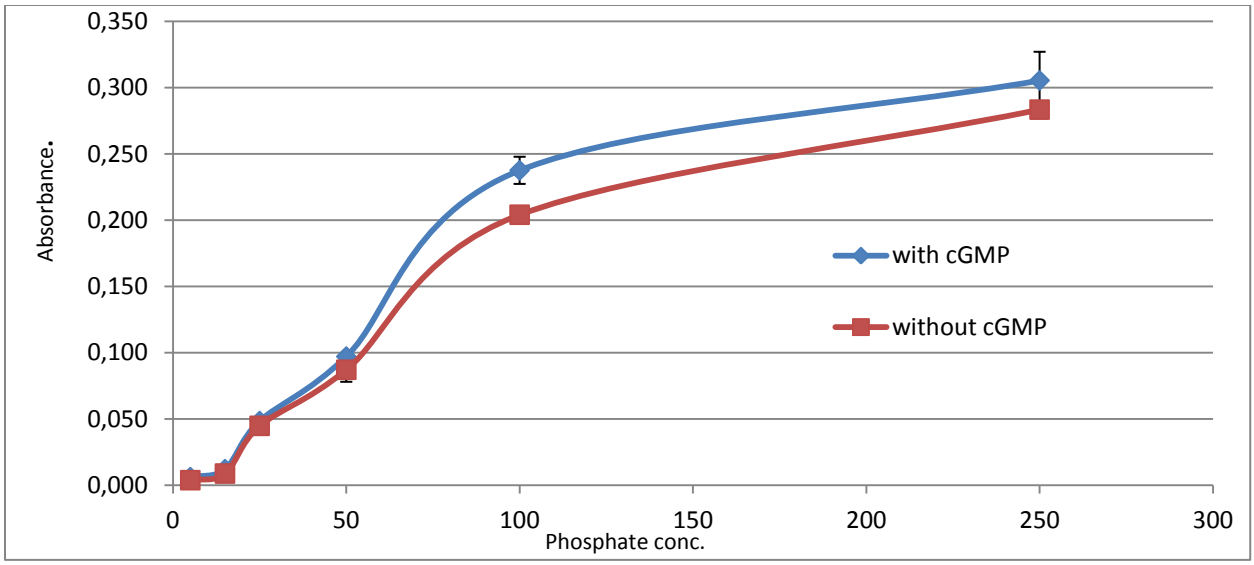


Table 2 and figure 7 shows that the phosphate concentrations appear to be higher in the presence of cGMP, probably due to degradation of cGMP with liberation of phosphate. The assays are virtually linear between 0 and 100 µM.

3.3. Phosphate assay and the effect of different concentrations of cGMP

Table 3: The table shows absorbance (at 680 nm) for different concentrations of cGMP and phosphate in the same concentrations to measure the color absorbance. For each concentration three parallels for cGMP and three parallels for same phosphate concentration were prepared. Absorbance measured at wavelength 680 nm for each parallel is represented by a, b, and c also calculated the mean and standard deviation for both (dilutions of phosphate concentration and dilutions of cGMP).

	Absorbance X 1000									
	With cGMP					With phosphate dilutions				
Dilutions Conc.µM	a	b	c	Mean	SD	a	b	C	Mean	SD
0.5µM	-7,0	-6,0	-9,0	-7,3	1,2	5,0	2,0	1,0	4,0	1,7
5 µM	-8,0	-9,0	-9,0	-10	2	3,0	2,0	3,0	3,0	0,5
25 µM	-8,0	-6,0	-9,0	-9,0	1,2	9,0	9,0	10,0	9,3	0,5
50 µM	-13,0	-7,0	-7,0	-9,0	0,9	21,0	21,0	22,0	21,3	0,5
100 µM	-9,0	-8,0	-8,0	-8,3	0,2	46,0	39,0	40,0	42,0	3,1

Figure 8: The figure shows the effect of cGMP and inorganic phosphate in identical concentrations on the absorbance. The values represent the mean values given in table 3.

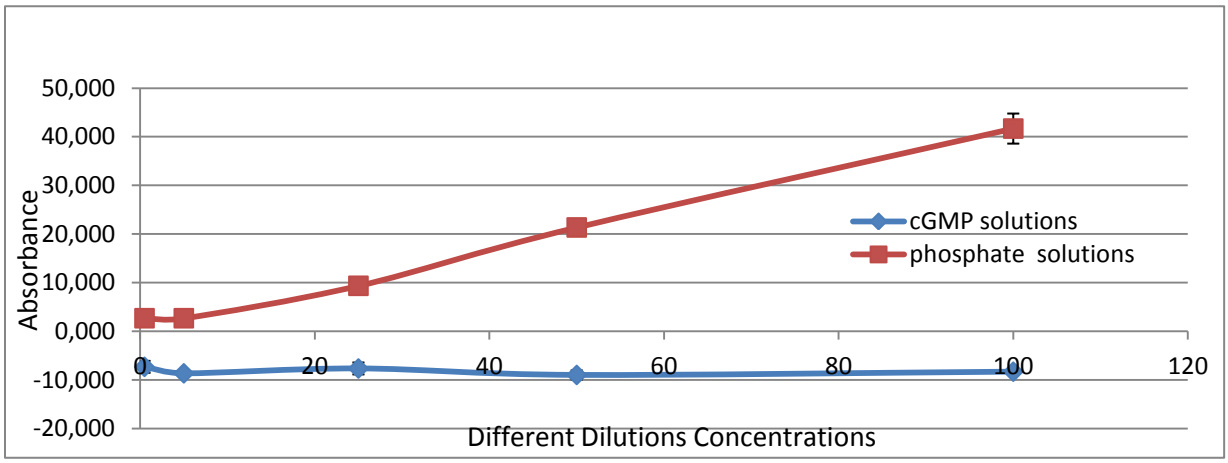


Figure 8 shows that the solutions of cGMP do not increase absorbance when present alone in the assay mixture.

3.4. Phosphate assay of IOV in presence and absence of cGMP

Table 4:

This table shows the absorbance obtained at 680 nm for IOV in absence and presence of 10 µM cGMP incubated for 60 min at 37°. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphate.

Sr.#	**Absorbance at 680nm x 100**	
	Without cGMP	**With cGMP**
1	7,6	7,7
2	8,1	7,8
3	7,6	8,1
4	8,0	8,3
5	8,0	8,1
6	7,9	8,1
7	7,5	8,0
8	7,8	8,0
9	8,0	8,0
10	8,2	8,5
11	8,4	8,6
12	8,1	8,7
13	8,6	8,5
14	8,5	8,5
15	8,5	8,9
16	9,9	10
17	7,6	9,9

Table 4 shows that 13 of 17 tests had higher phosphate level in presence of cGMP, compatible with a cGMP-stimulated ATPase, whereas two were higher without cGMP addition and two were not different.

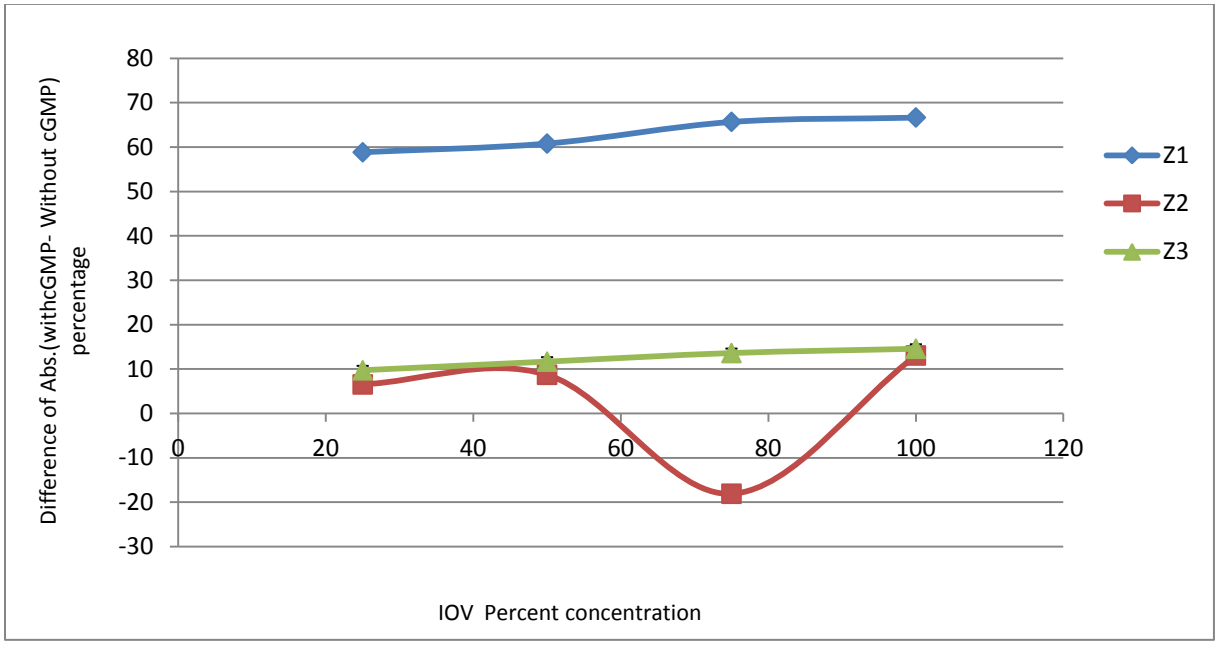
3.5. Effect of different IOV concentrations on ATPase assay:

A linear increase in absorbance would be expected with increasing IOV concentrations. IOV in absence and presence of cGMP were tested three times, following same protocol.

Table 5: The table shows difference of absorbance (at 680 nm) in percent for different concentrations of IOV from three different experiments, represented as Exp1, Exp 2 and Exp 3.

IOV conc. %	Difference of absorbance at 680nm (%) (abs. with cGMP – abs. without cGMP) / Max Abs.) x 100		
	Exp 1 (%)	Exp 2 (%)	Exp 3 (%)
25	58,8	6,5	9,7
50	60,8	8,7	11,7
75	65,7	-18,1	13,6
100	66,7	13,0	14,6

Figure 9: The figure shows three independent experiments with cGMP-stimulated ATPase activity as function of IOV concentration. The three curves represent Exp1 (Z1), Exp2 (Z2) and Exp3 (Z3).



With the exception of the third point in experiment Z2 all experimental points indicate an IOV concentration-dependent increase in phosphate production. However, the specific activity (activity above basal) is very low in experiment Z2 and Z3.

3.6. Difference in absorbance in presence and absence of IOV

These experiments were performed to measure the background absorbance (reagent blank) and determine whether IOV gave additional increase in absorbance.

Table 6: The table shows the optical absorbance x100 obtained at 680nm at 40 minutes for two different samples with and without IOV in the absence of cGMP. The samples were incubated for 60 min at 37°C. Reaction was stopped and OD was measured.

	With IOV	**Without IOV**
Absorbance x 100	13.0	10.0
	13.0	9.0
	13.1	9.0
Mean Absorbance	13.0	9.3

The experiments were conducted to demonstrate that the reaction mixture with IOV gave higher phosphate levels than without. Table 6 shows a small but distinctive increased absorbance when IOV were present. This represents basal ATPase activity. The absorbance without IOV represents the reagent blank.

3.7. ATPase assay with higher IOV concentrations

These experiments were performed to see whether higher concentrations of vesicles gave higher specific activity (the difference in absorbance with cGMP and without cGMP). The IOV-stock used here were less diluted than before, i.e. 6 times more concentrated

Table7: The table shows the difference of absorbance (at 680 nm at 40 min) in percent for different concentrations of IOV from three different experiments, represented as Exp1, Exp 2 and Exp 3.

IOV conc. %	Difference of absorbance at 680nm In percent (abs. with cGMP – abs. without cGMP) / Max Abs.) x 100		
X	Exp 1 (%)	Exp 2(%)	Exp 3 (%)
25	5,3	80,9	1,7
50	1,1	66,0	13,6
75	29,8	76,6	6,8
100	42.6	63,8	8,5

The results of these experiments (table 7) are not consistent and the expected linear increase in absorbance as a function of IOV concentration can not be seen.

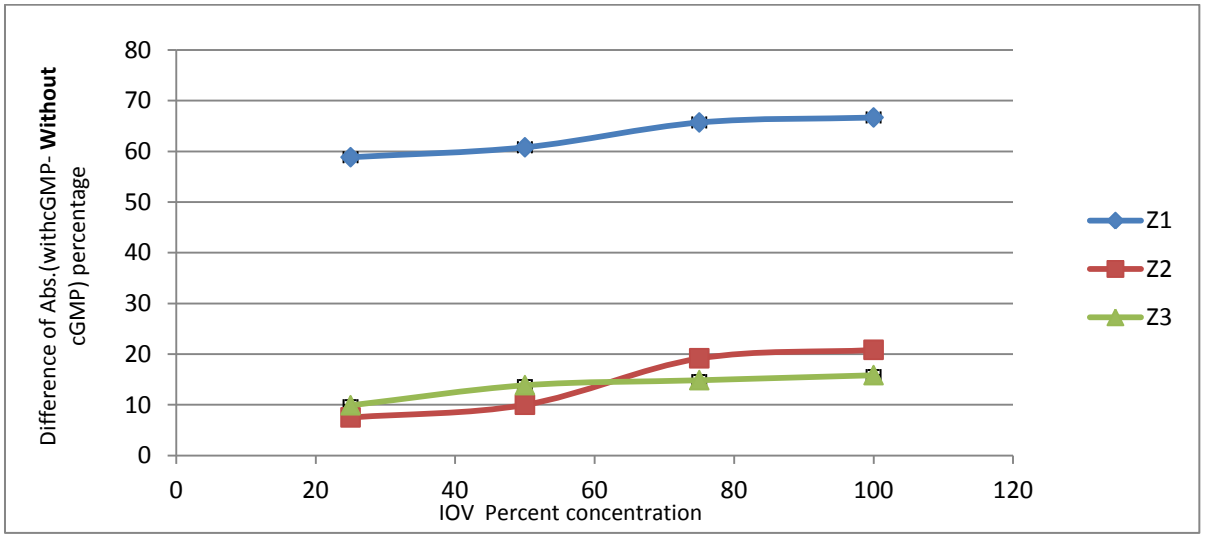
3.8. ATPase assay with higher IOV concentrations with modified protocol

Based on the unsatisfactory results in section 3.7 (table 7) the protocol was modified (see Appendix 7.5.8) and for these experiments not only 6 times concentrated IOV- stock was used but also decided to remove maximum possible phospholipids before adding color reagent so that absorbance recorded should be of only inorganic phosphate released during ATPase.

Table 8: The table shows the difference of absorbance, the specific cGMP stimulated activity (with 10 µM cGMP for 60 min incubation at 37°C) in percent for different concentrations of IOV from three different experiments, represented as Exp1, Exp 2 and Exp 3.

IOV conc. %	Difference of absorbance at 680 nm (%) (abs. with cGMP – abs. without cGMP) / Max Abs.) x 100		
X	Z1 (%)	Z2 (%)	Z3 (%)
25	58,8	7,5	9,9
50	60,8	10,0	13,9
75	65,7	19,2	14,9
100	66,7	20,8	15,8

Figure 10: The figure shows IOV concentration along X-axis and difference of absorbance in percent (Z) along Y-axis. Three curves representing Z1, Z2 and Z3 for Exp 1, Exp 2, and Exp 3 respectively, are following linear increase behavior.



After modification of the protocol the expected results occurred. The cGMP-stimulated ATPase activity increased as a function of IOV concentration. However, Exp1 showed clearly higher specific activity than Exp 2 and Exp 3, which were grouped together. In a more detailed analysis of the single experiments it was shown that trend lines for Exp1, Exp2 and Exp3 were described by y = 0,1137x + 55,88 (R^2 = 0,9397), y = 0,1966x + 2,085 (R^2 = 0,9222) and y =0,0752x + 8,91 (R^2 = 0,8699), respectively. This data suggests that linearity exists.

3.9. ATPase assay of IOV with commercial kit

The following experiments were undertaken to obtain experience with a commercial ATPase kit (Innova Biosciences).

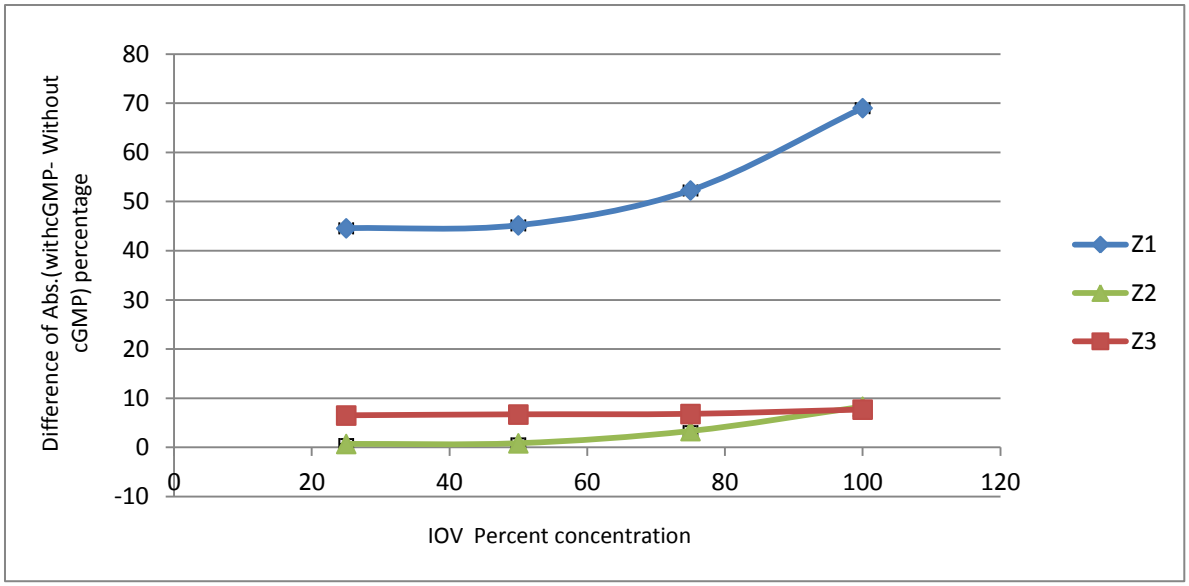
Table 9:

The table shows difference of absorbance (at 635 nm) in percent for different concentrations of IOV from three different experiments, represented as Exp1, Exp 2 and Exp 3.

IOV conc. %	Difference of absorbance at 635 nm In percent (abs. with cGMP – abs. without cGMP) / Max Abs.) x 100		
	Exp 1 (%)	Exp 2 (%)	Exp 3 (%)
25	44,6	0,7	6,5
50	45,2	0,9	6,7
75	52,3	3,3	6,8
100	69,0	8,3	7,7

Figure 11:

The figure shows the difference of absorbance in percent along Y-axis as a function of IOV concentration. Three curves representing Z1, Z2 and Z3 for Exp 1, Exp 2, Exp 3, are showing linear increase and are following more significant increasing pattern



The commercial kit gave results that showed a trend of relation between phosphate concentration and % IOV, The cGMP-stimulated ATPase activity increased as a function of IOV concentration. However, Exp 1 showed clearly higher specific activity than Exp 2 and Exp 3, which were grouped together. In a more detailed analysis of the single experiments it was shown that trend lines for Exp 1, Exp 2 and Exp 3 were described by $y = 0{,}3218x + 32{,}655$ ($R^2 = 0{,}8312$), $y = 0{,}101x - 3{,}015$ ($R^2 = 0{,}846$) and $y = 0{,}0166x + 5{,}91$ ($R^2 = 0{,}9153$), respectively. These data may indicate that linearity exists.

4. Discussion

The current study was conducted to characterize cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes. As described earlier, one class of ATPases utilizes stored energy (ATP) to produce useful work against concentration gradients e.g. m-ATPase or ABC-transporters. [33] These are primary active transporters which require ATP hydrolysis for translocation of substance across cell membrane.

In recent years much work has been done regarding ABC (ATP-Binding-Cassette) transporters. In almost all living cells the members of this transporter family have been identified. [33, 5] Pharmacological studies of cyclic nucleotide extrusion confirmed, human erythrocytes (hRBC) which possess ABCC4 [16, 20] and ABCC5 [14, 16, 7, 4] a suitable choice. [23] We believe that ABCC4 is a selective high affinity transporter for cAMP whereas ABCC5 is selective high affinity transporter for cGMP [23] but that both proteins transport both nucleotides with low affinity. [33] Previous studies support the concept that cGMP transporter also requires lipid environment for ATPase activity like other membrane bound protein ATPases. [4]

There are biochemical evidences available explaining the functions of cGMP efflux pump, showing transport is dependent on ATP [21] and ATP hydrolysis [23] with magnesium as activator. [26, 27, 5]

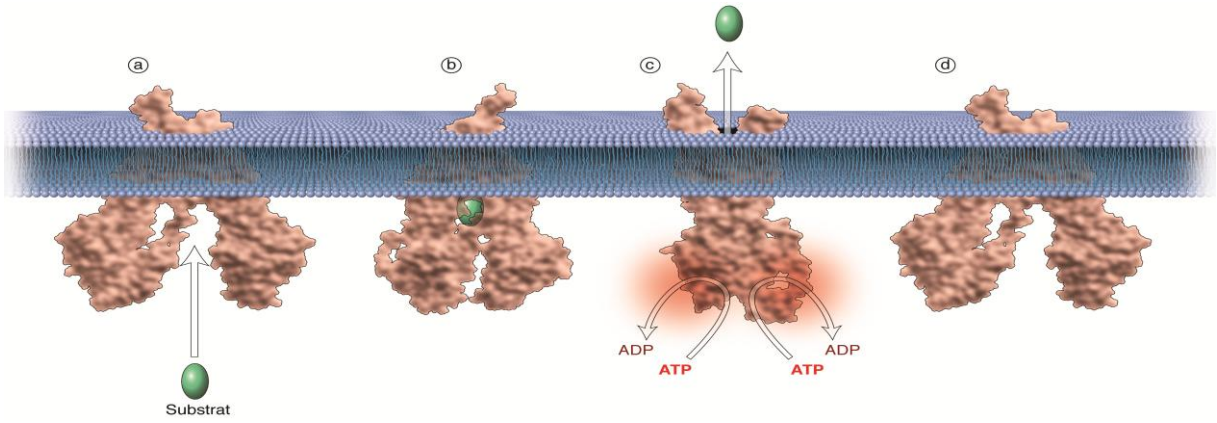


Figure 12: ABCC5 Transporter and ATPase activity (ill.: Roy Lysaa).

A: Inward opening of the transporter domain. **B:** Substrate binding causing conformational change. **C:** ATP binding → separation of the membrane-binding domains → Substrate release to the outward → ATP hydrolysis. **D:** Transporter is resettled to its initial.

In present study we have characterized an assay for determination of inorganic phosphate concentrations and employed this method to study basal and cGMP- stimulated ATPase activity in IOV from hRBC.

The project was divided in three parts

1. Preparation of IOV from hRBC.
2. Characterization of ATPase activity in IOV of hRBC after stimulation with cGMP, aimed to design in-house assay method for.
    - Test of linearity of inorganic phosphate standard curve.
    - Determine the influence of membrane protein concentrations on the linearity of the inorganic phosphate assay (dilution studies).
    - The ability to distinguish basal ATPase activity from cGMP-stimulated activity in hRBC IOV and finally,
3. Compare the In-house assay with commercially available kit (Innova Biosciences) to make cost beneficial analysis.

4.1. Preparation of IOV from hRBC and sidedness test:

The first part of the project was to prepare IOV from hRBC.

As known from previous studies biological membrane have differential two compartment behavior because of the different molecular constituents of both surfaces. The principal feature of membrane organization is this asymmetry.

To characterize the individual surfaces behavior suggestion was to prepare membranes, sealed into vesicles of one specific orientation by following step by step approach. [25]

So for present study three different batches of inside out vesicles (IOV) were prepared from freshly drawn human blood and tested for sidedness.

As mentioned in chapter RESULTS (3.1) three different percentage yields of IOV were obtained.

The percentage yield of IOV is presented below:

- From batch 1 was 50%
- From batch 2 was 26%
- From batch 3 was 28%.

The first batch produced comparative good yield of 50% IOV where as in 2nd and 3rd batch yield was almost half of the 1st value. This varying percentage yield of IOV may have different explanations.

- Incubation time in IOV permeable buffer varied, in first batch it was exactly according to protocol only 2 hours. But in other cases due to the length of experiment, ghosts left incubated overnight.
- Another possible reason could be any possible contamination causing change in pH resulting IOV permeability behavior of incubating buffer.
- There was also possibility of movement even minor shaking could result in disturbing of two phase layers while doing phase separation after treating with density gradient.

Following proposals are recommended for future investigations.

- Incubating time and temperature should be constant and according to the protocol.
- Handle two phase density gradient layers gently but with high stability while separating two phases.
- Recommended to use freshly prepared incubating buffer to avoid pH variation.

4.2. Characterization of cGMP stimulated ATPase activity in IOV of hRBC

The second step of the project was to characterize ATPase activity in IOV of hRBC after stimulation with cGMP.

The idea was to design an in-house assay method for the determination of inorganic phosphate concentrations and to employ this method for basal and cGMP stimulated ATPase activity in IOV from hRBC.

It was in 1925 Fiske and Subbarow used colorimetric technique for determination of phosphomolybdate complexes since then many modifications have been made and now it is widely used biochemical technique. [1, 2, 10, and 13]

Further investigations confirmed that the ATPase activity is determined by measuring the release of inorganic phosphate generated from ATP hydrolysis by colorimetry. ATPase activity for IOVof human erythrocytes is a function of substrate binding and leads to substrate translocation by the ABC-transporter. In current studies a modified assay method based on the work of Chifflet and Boadu & Sager [6, 3] was designed using principle elements,

a) **Transporter:** Human erythrocytes contain ABCC5 transporters which are suitable for measuring cGMP derived ATP hydrolysis.
b) **Substrate:** Transported substrate regulates the ATPase activities of several transporters, for stimulating the ATPase activity of the cGMP transporter, cGMP was used. [3] To avoid competing low-affinity transporters, it is recommended to use not more than 10 µM concentration of cGMP.
c) **Inhibitors:** Since there is a number of other ATPases in the cell, these have to be blocked by specific inhibitors.
    - Na^+/ K^+-ATPase is inhibited by 1 mM Ouabain.
    - Ca^{2+}-ATPase is inhibited by 2 mM EGTA.

This part of the project was aimed to,

1. Test the linearity of the inorganic phosphate standard curve.
2. Determine the influence of membrane protein concentrations on the linearity of the inorganic phosphate assay (dilution studies).
3. The ability to distinguish cGMP-stimulated activity from basal ATPase activity in hRBC IOV.

4.2.1. Test the linearity of the inorganic phosphate standard curve

In biological systems phosphate is widely available, multifunctional inorganic ion which plays important role in regulating protein kinase and phosphatases dependent enzyme activity. Previous studies suggest that there is significant linear relation between amount of phosphate released and absorbance measured. To test this linear behavior, initially a phosphate assay was conducted using six different phosphate concentrations (5 µM, 15 µM, 25 µM, 50 µM, 100 µM, and 250 µM) which were incubated at 37 ºC in solution with and without cGMP to optimize activity and colorimetric analysis resulted that in the presence of cGMP phosphate concentrations tend to have higher absorbance probably due to cGMP degradation as shown in table 2 figure 7. This difference was much prominent with 25 µM and higher phosphate concentration and appeared to be virtually linear between 0 and 100 µM, so it was decided to ignore phosphate concentrations less than 25 µM in next series of experiments. Phosphate concentration of sample can also be determined by formula

$$\text{Phosphate Concentration} = \frac{\text{(sample abs. – blank abs.)}}{\text{(slope of standard curve) x (µl of sample)}}$$

During an ATPase activity to justify the role of cGMP and inorganic phosphate in color complex formation, a phosphate assay for different concentrations of cGMP, and phosphate solutions of same concentrations was performed. Amount of cGMP is replaced with water in incubation solution. Dilution series (0.5 µM, 5 µM, 25 µM, 55 µM, 100 µM) for same concentrations of phosphate solution and cGMP solutions are prepared and analysed following the protocol. The results of this experiment presented in table 3 and figure 8 showed, during phosphate assay, cGMP has no role in color development. Color development is due to phosphate released during incubation time. So testing the linearity of the inorganic phosphate standard curve concluded that the phosphate assay is linear but influenced by the presence of cGMP and color development is a function of phosphates released during the assay.

As described earlier colorimetric determination of inorganic phosphate as a result of ATP hydrolysis is a function of ATPase activity in any biological system. This assay method was decided to use for further more ATPase analysis of IOV from human erythrocytes.

4.2.2. Influence of membrane protein concentrations (dilution studies)

In Pharmacological studies of biological membrane proteins, for assaying substrate transport in primary transport systems, IOV are first choice as supply of ATP to the transport system is exofacial in IOV, which provide the driving force for substrate transport. [28].

To figure out the influence of membrane proteins concentrations it is expected that with increasing IOV concentrations there would be a linear increase in absorbance measured. Keeping this in mind to test the effect of different IOV concentrations an ATPase assay for IOV was performed, using phosphate assay method where concentrations of phosphate are replaced with concentrations of IOV with vesicles dilution of 25 %, 50 %, 75 %, 100 % and incubated at 37 ºC in the solution with and without cGMP to optimize activity. For each IOV concentration e.g. 25 % three parallels with cGMP and three without cGMP were prepared to avoid possible errors also measured the optical absorbance (OD) at 40 minutes and wavelength 680 nm. For the precise results this experiment following the same protocol was performed three times. For each experiment protein concentration of vesicles was different as for each experiment 1ml aliquot of pre frozen IOV suspension was thawed and used for making dilutions so for analyzing the data obtained, the difference of absorbance in percent was calculated and plotted against IOV concentrations in table 5 and figure 9. The curves

obtained from experiment 1 and 3 showed an increasing linear behavior in terms of increasing IOV concentrations where as experiment 2 does not fully supports the concept rather showing negative value at 75 % IOV concentration. The explanation for this is not clear and with the exception of this particular point all experimental points indicates an IOV concentration – dependent increase in phosphate production.

During behavior studies of influence of membrane protein concentrations, the effect of higher IOV concentrations was a question of interest apparently suggested that higher concentrations of inside out vesicles will give higher specific activity so that new experiments were performed by six times concentrating the IOV using centrifugation technique and this concentrated IOV stock suspension was used to make (25 %, 50 %, 75 % and 100 %) dilution series. Rest of the procedure was unchanged. Final data obtained is shown in table 7. The results of these experiments are not consistent and expected linear increase in absorbance as a function of IOV concentrations is absent. This finding suggested that might IOV are also playing some role in color development activity and may be possible cause of this strange non-linear pattern. Although lipid environment supports the ATPase activity but to verify the catalytic role of phospholipids presents in IOV and to limit their activity in color development, is a function of determination of free inorganic phosphate released during ATPase. So based on unsatisfactory results from above mentioned experiment the protocol was modified and the suggestions were; use of concentrated IOV stock suspension, removal of maximum possible phospholipids before adding color reagent and increasing the color reaction time. Next experiments with slight modifications in protocol were performed. The modifications made in protocol are as below,

- Used six times concentrated IOV stock suspension involving centrifugation technique to make further vesicle dilution of 25 %, 50 %, 75 %, and 100 % for confirming maximum amount of IOV per dilution concentration.
- After incubation times is over, all the samples with or without cGMP were centrifuged at 13000 RPM for 15 min to get rid most of the phospholipids.
- In last, optical absorbance (OD) measured at 680 nm wavelength and at 60 minutes believing that increased reaction time for color development might give more significant absorbance value.

After modification of the protocol expected results occurred as shown is in table 8 and figure 10. All three curves for experiment 1, 2 and 3 are showing increase cGMP stimulated ATPase activity. In more detailed analysis of the single experiment it was shown that trend lines for

Exp 1, Exp 2 and Exp 3 were described by y = 0,1137x + 55,88 (R^2 = 0,9397), y = 0,1966x + 2,085 (R^2 = 0,9222) and y =0,0752x + 8,91 (R^2 = 0,8699), respectively. This data suggested that linearity exists and concluded that Increasing dilution series of IOV, stimulated with cGMP have relative increasing ATPase-activity. The amount of IOV present in a particular concentration is directly proportional to absorbance, and plasma membrane lipids (phospholipids) present in IOV of human RBC are responsible for modulation of the catalytic activity of cGMP dependent ATPase.

4.2.3. The ability to distinguish cGMP stimulated ATPase from basal ATPase

To confirm the behavior of hRBC inside out vesicles in term of the ability to distinguish cGMP stimulated activity from basal ATPase activity following experiment was conducted.

For measuring cGMP stimulated ATPase activity of IOV, phosphate assay of IOV in presence and absence of cGMP was performed where phosphate different concentrations were replaced with same concentration of IOV. Seventeen parallels were prepared and OD at 40 minutes was measured to get relative clear picture results obtained are presented in table 4 shows that 13 of 17 tests had higher phosphate level in presence of cGMP, compatible with a cGMP-stimulated ATPase, whereas two (sample 2 and 13) were higher without cGMP addition and two were not different. Overall IOV in the presence of cGMP have increase ATPase activity.

While talking about basal ATPase activity of IOV, without cGMP stimulation, the difference in absorbance in presence and absence of IOV is an important consideration. An experiment was performed to measure absorbance of reagent blank (without IOV) and comparing with absorbance of IOV to determine whether IOV gave additional increase in absorbance in the absence of cGMP stimulus. Two analytes were prepared with and without IOV in the absence of cGMP following the assay procedure, optical absorbance at 680 nm was measured. Observations were shown in Table 6 resulted, the reaction mixture with IOV gave higher phosphate levels than without, by a small but distinctive increased absorbance without cGMP. The findings supported the concept that hRBC IOV shows basal ATPase activity in the absence of cGMP which is might due to phospholipids presents in IOV and may be these phospholipids are playing role in color development. Removal of most phospholipids after stimulation with cGMP incubation would possibly decrease external phosphates available for color complex formation during ATPase assay. Finally concluding that IOV of human

erythrocytes posses basal ATPase activity in the absence of cGMP where as cGMP stimulated IOV of hRBC have comparative higher ATPase activity.

At this point we concluded to perform further experiment for the 2nd part of the project "to characterize ATPase activity after stimulation with cGMP". Experimental work done for this study lead us to design an assay method which can be utilize as an in-house ATPase assay and is a modified version of method based on the work of Chifflet and Boadu & Sager. [6, 3] Summarizing the second part of the project, key points are,

- The phosphate assay is linear but influenced by the presence of cGMP.
- Color development is a function of phosphates released during assay activity.
- Increasing dilution series of IOV, stimulated with cGMP have relative increasing ATPase activity.
- The amount of IOV present in a particular concentration is directly proportional to absorbance.
- IOV of human erythrocytes have basal ATPase activity in the absence of cGMP where as cGMP stimulated IOV of hRBC have comparative higher ATPase activity.
- Plasma membrane lipids (phospholipids) present in inside out vesicles of human RBC are responsible for modulation of the catalytic activity of the cGMP dependent ATPase.

4.2.4. ATPase Assay by commercially available kit:

An experiment was set for measuring ATPase activity in IOV of human erythrocytes by ATPase commercially available kit. For this study ATPase Assay kit by **Innova Biosciences** was recommended. The kit is provided with P_iColorLock™ Gold reagent (an improved malachite green formulation) with additives to prevent background signals arising out of non-enzymatic ATP hydrolysis. According to the instructions by supplier and following the procedure protocol, assay was performed and optical density at 635 nm and was recorded.

After data analysis final observations are shown in table 9 and figure 11, a trend of relation between phosphate concentrations and % IOV, the cGMP-stimulated ATPase activity increased as a function of IOV concentration. However, In a more detailed analysis of the single experiments it was shown that trend lines for Exp1, Exp 2 and Exp 3 were described by $y = 0{,}3218x + 32{,}655$ ($R^2 = 0{,}8312$), $y = 0{,}101x - 3{,}015$ ($R^2 = 0{,}846$) and $y = 0{,}0166x + 5{,}91$ ($R^2 = 0{,}9153$), respectively. These data may indicate that linearity exists but the values of

R^2 in case of in-house ATPase assay method were more close to 1 than that of Innova Biosciences ATPase assay kit.

4.3. Comparing In-house assay with commercial ATPase assay kit:

As mentioned before in aims of work last part of the current study was to compare, in-house assay method with commercially available ATPase assay kit to make cost benefit analysis. For this purpose above mentioned ATPase Assay kit from Innova Biosciences was used and compared under following parameters.

a. Authenticity / Reliability of method
b. Precision and accuracy of results
c. Time to get result
d. Cost.
e. Others (if there, mention it).

Findings, after comparing both methods for above mentioned parameters are discussed in briefs as below.

Authenticity / Reliability of method:

While talking about authenticity of methods, it is believed that commercially available assay kit is more reliable as chemicals and reagents are already prepared of required concentrations. pH is sustained for reagents and for each pack. Finished ready to use substrate, buffers, accelerator and inhibitors are available in same kit and only few steps are required to get results making it easier to perform the experiment. Whereas for in-house assay method, one needs to prepare all solutions and reagents before performing experiment of required concentrations and pH. Stability of pH is difficult to obtain depending upon storage conditions, and human errors e.g. (weighing, pippeting and handling errors, missing / addition of any ingredient, environmental contamination, temperature variations etc). Lots of the steps are involved from A- Z for each experiment to get results making it complicated.

Precision and accuracy of results:

Precision and accuracy of results is core for any study and votes for method of choice to perform experiments and helps to draw the conclusion. In current study the results obtained from in-house ATPase assay method were more accurate and precise. Sensitive design of the

commercial kit could not get extra advantage although all the reagents were packed and sealed in small quantities sufficient for once use, keeping it safe from decaying or contaminating. But careful preparation of all reagents and solutions, proper pippeting technique, avoiding maximum possible human errors, best storage conditions, pH stability and favorable environmental conditions maintained during In-house ATPase assay procedure provided best reaction conditions for maximum ATPase activity, resulting in more precise measurements in contrast to commercial ATPase assay.

Time to get result:

During any scientific work the time used to get significant results is also important factor and aids for a valuable conclusion. In present scientific work done for measuring, cyclic nucleotide dependent ATPase activity in IOV of hRBC by In-house designed ATPase assay method it took almost continuous seven hours to get significant values for each set of experiment as compared to 3 - 4 hours used for measuring ATPase activity of IOV from hRBC while stimulating with cGMP by commercially available Innova Biosciences ATPase assay kit.

Cost

Economy is a key factor for method of choice for any scientific work. In-house assay method for measuring ATPase activity is highly cost effective. Almost all chemicals to make solutions and reagents are easily available in bulks in labs and one can prepare solutions of choice in good quantity and in good price comparing with commercially available kit which is rather expensive.

Others:

Effective scientific method is always reproducible. This key factor is a big support for in-house method designed to measure ATPase activity of IOV from hRBC. To repeat the experiment in case of spillage or as per need is quite easy and possible. The availability of required information about all the chemicals and reagents and complete protocol to prepare solutions and buffers made it possible to reproduce it any time and in any part of the world, if you do not have access to commercially available ATPase assay kit of Innova Biosciences and can justify experimental findings.

Summarizing, for an analysis, using commercially available ATPase kit by Innova Biosciences is comparative expensive but this assay technique is time saving and easy to use.

In-house ATPase assay method is not only cost effective but also has good reproducibility although takes little longer time than commercial assay kit. This assay method is not limited as in case of future investigations, modification could be made by addition or removal of any reagent, changing the concentration or pH of any solution etc.

5. Conclusions:

Present study was conducted to measure, "Cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes" by an in-house assay method and to compare it with commercial ATPase assay kit to make a cost benefit analysis.

Present data obtained from experimental work shows that we have been able to design an assay method to measure cGMP dependent ATPase activity as an in-house assay method and we also compared it with commercially available ATPase assay kit by **Innova Biosciences**.

The main findings were:

1) The inorganic phosphate standard curves were linear for relevant biological concentrations, observed for both in-house and commercial assay.

2) The membrane protein (i.e. IOV) concentrations raised the inorganic phosphate concentrations linearly.

3) It was possible to distinguish cGMP-stimulated activity from basal ATPase activity in hRBC IOV, even though the difference was small.

4) The two phosphate assays (in-house and commercial) had both advantages and disadvantages, none being superior to the other one.

5.1. Future directions:

A scientific work always leaves open doors behind for new findings. So it is in this case as well. Some proposals for future investigations of cyclic nucleotide dependent ATPase activity in inside out vesicles from human erythrocytes could be,

- Use different incubation time (longer or shorter).
- By stimulating with different concentrations of cGMP less than 10µM.
- Comparing with any other commercially available kit.
- ^{33}P-ATP-method.

6. Literature:

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7. Appendix:

7.1. List of chemicals:

Description	Components	Notes (store at 4ºC if not otherwise noted)
NaCl	Sodium chloride	≈ saturated, keep at room temp.
KCl	Potassium chloride.	≈ saturated, keep at room temp.
KOH	Potassium hydroxide.	Store in plastic bottle, keep at room temp.
EGTA•$2K^+$	(-$CH_2OCH_2CH_2N(CH_2CO_2H_2)_2$	Neutralize with KOH
HEPES	HEPES:$HOCH_2CH_2$-N(-C_2H_2-)$_2$N-$CH_2CH_2SO_3H$ HEPES potassium salt: $HOCH_2CH_2$-N(-C_2H_2-)$_2$N-$CH_2CH_2SO_3K$	Gives pH =7.50 at osm = 300
Magnesium chloride	$MgCl_2$•$6H_2O$	≈ saturated, keep at room temp.
ATP	Na_2ATP	Keep ice cold on bench, store at -70°C
Phosphate, basic	Na_2HPO_4•$2H_2O$	
Phosphate, acidic	NaH_2PO_4•H_2O	
Arsenate	Na_2HAsO_4•$7H_2O$	Poisonous
Pyrophosphate	$Na_4P_2O_7$•$10H_2O$	
HCl (acid)	37 % Hydrochloric acid	P = 1.184 g/ml
NaOH (base)	NaOH	
Protease inhibitors	Protease inhibitor cocktail	SigmaP2714 Use as is.
Cysteine	Cysteine $C_3H_7NO_2S$	store in glass bottle, dark & cool in inert N_2-atmosphere
NAD	β-Nicotinamide adenine dinucleotide	Keep ice cold on bench, store at -70°C.

ATCCs	Acetylthiocholine-chloride	Keep ice cold on bench, store at -70°C.
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)	
Histodenz	Histodenz	Sigma D 2158
G-3-P	Glyceraldehyd-3-phosphate	Keep ice cold on bench, store at -70ºC, use as is. Sigma D5251
Triton X-100	Triton X-100	
Tris.	$(HOCH_2)_3CNH_2$	Store in cooling room.
Tris concentrated, acidic	Tris•HCl	Store in cooling room.
Potassium phosphate, acidic	KH_2PO_4	
Potassium phosphate basic	$K_2HPO_4•3H_2O$	
cGMP	cyclic guanosine--monophosphate	Keep ice cold on bench, store at s-70°C.
Sodium dodecyl sulphate	Sodium dodecyl sulphate	
Ouabain	g-strophanthin $C_{29}H_{44}O_{12}$	**Very Poisonous!!** Store in freezer.
Ammonium molybdate	Ammonium molybdate $(NH_4)_2MoO_4$	
Ascorbic acid.	$C_6H_8O_6$	

7.2. Buffers and solutions:

Name	Description	Amoun-ts	Components	Water is added until final vol.(ml)	Final molarity of main components	Notes (store at 4ºC if not otherwise noted)
NK	NaCl	29.22 g	NaCl	100	NaCl 5000	≈ saturated, keep at room temp.
KK	KCl	29.82 g	KCl	100	KCl 4000	≈ saturated, keep at room temp.
KOH	KOH	28.06 g	KOH	100	KOH 5000	Store in plastic bottle, keep at room temp.
EGN	EGTA•$2K^+$, Neutral	6.168 g 8000 µl	(-$CH_2OCH_2CH_2N(CH_2CO_2H_2)_2$ **KOH.**	100	EGTA 200 K^+ 400	Neutralize with KOH
HEPK	HEPES	25.50 g 25.65 g	HEPES:$HOCH_2CH_2$-N(-C_2H_2-)$_2$N-$CH_2CH_2SO_3H$ HEPES potassium salt: $HOCH_2CH_2$-N(-C_2H_2-)$_2$N-$CH_2CH_2SO_3K$	200	HEPES 1000 K^+ 464	Gives pH =7.50 at osm = 300
MG	Magnesiumchloride	5.083 g	$MgCl_2$•$6H_2O$	25	$MgCl_2$ 1000	≈ saturated, keep at room temp.
ATP	ATP	3.31 g	Na_2ATP	20	NA_2ATP 300	Keep ice cold on bench, store at -70°C
CV	Cell washing	10000 µl 10000 µl 30000 µl	**HEPK** **EGN** **NK**	1000	HEPES 10 K^+ 8.6 EGTA 2 NaCl 150	pH = 7.50
L1	Lysis buffer # 1	5000 µl 4000 µl 4000 µl 40000 µl	**EGN** **MG** **ATP** **HEPK**	2000	EGTA 0.5 $MgCl_2$ 2.0 NA_2ATP 0.6 HEPES 20.0 K^+ 28.9	pH = 7.50
L2	Lysis buffer # 2	5000 µl 4000 µl	**EGN** **HEPK**	2000	EGTA 0.5 HEPES 20 K^+ 28.9	pH = 7.50
FB	Phosphate, basic	89.00 g	Na_2HPO_4•$2H_2O$	1000	Phosphate 500	
FS	Phosphate, acidic	13.80 g	NaH_2PO_4•H_2O	500	Phosphate 200	
AK	Arsenate	15.60 g	Na_2HAsO_4•$7H_2O$	50	Arsenate 1000	Poisonous
PK	Pyrophosphate	22.30 g	$Na_4P_2O_7$•$10H_2O$	250	Pyrophosphate 200	

HC	HCl (acid)	9.858 g	37 % hydrochloric acid	100	HCl 1000	P = 1.184 g/ml
NOH	NaOH (base)	0.200 g	Sodium hydroxide	50	NaOH 100	
PI	Protease inhibitors	1 bottle	Protease inhibitor cocktail	100		SigmaP2714 Use as is
C	Cysteine	0.0606g 3900 µl	Cysteine **NOH**	100	Cysteine 5	pH = 8.40 store in glass bottle, dark & cool in inert N_2-atmosphere
NAD1	NAD solution # 1	0.183 g 13750 µl	β-Nicotinamide adenine dinucleotide Water		NAD 20	Keep ice cold on bench, store at -70°C.
ATCC	ATCC solution	0.0250g 10000 µl	Acetylthiocholine-chloride Water		ATCC 12.5	Keep ice cold on bench, store at -70°C.
IS2	Incubation solution # 2	0.0139g 3880 µl 8450 µl	5,5'-dithiobis-(2-nitrobenzoic acid) **FS** **FB**	50	DTNB 0.7 Phosphate 100	pH = 7.50
NAD2	NAD-solution #2	3750 µl 6250 µl	**NAD1** Water		NAD 7.5	Keep ice cold on bench, store at -70ºC.
T5	Density # 5	28.27 g 1424 µl 1430 µl 68 µl	Histodenz **FB** **FS** **KK**	100	Phosphate 10 Histodenz 28.27%	pH = 7.50 p =1.150 Histodenz; Sigma D 2158
G3PK	G-3-P, concentrated	0.100 g	Glyceraldehyd-3-phosphate	2	Glyceraldehyde3-phosphate 294	Keep ice cold on bench, store at -70ºC. Use as is. Sigma D5251
G3P	G-3-P	204 µl 890 µl 2906 µl	**G3PK** **NOH** Water		Glyceraldehyde3-phosphate 15	pH = 7.00 Keep ice cold on bench, store at -70ºC
PBS	Phosphate Buffered Saline	15800 µl 10000 µl 27400 µl 675 µl	**FB** **FS** **NK** **KK**	1000	Phosphate 10 NaCl 137 KCl 2.7	pH = 7.40
PA	Pyrophosphate arsenate buffer	3750 µl 1500 µl 340 µl	**PK** **AK** **H**	50	Pyrophosphate 15 Arsenate 30	pH = 8.50 Poisonous
P	Pyrophosphate buffer	7500 µl 735 µl	**PK** **HC**	50	Pyrophosphate 30	pH = 8.40
A	Arsenate solution	4000 µl	**AK**	10	Arsenate 400	Poisonous
T4	Density #4	5580 µl 420 µl	**T5** **PBS**		Histodenz 26.3%	pH = 7.40 p = 1.140
T3	Density # 3	5170 µl 830 µl	**T5** **PBS**		Histodenz 24.3%	pH = 7.40 p = 1.130

T2	Density # 2	4750 µl 1250 µl	**T5** **PBS**		Histodenz 22.4%	pH = 7.40 p = 1.120
T1	Density #1	21670 µl 8330 µl	**T5** **PBS**		Histodenz 20.4%	pH = 7.40 p = 1.110
T0	Density #0	11750 µl 6250 µl	**T5** **PBS**		Histodenz 18.5%	pH = 7.40 p = 1.100
F0	Phosphate buffer with 0 detergent	125 µl 448 µl	**FS** **FB**	50	Phosphate 5	pH = 8.00
FX	Phosphate buffer with Triton X-100	25000 µl 50 µl	**F0** Triton X-100		Phosphate 5 Triton X-100 0.2%	pH = 8.00
IS1	Incubation solution for sidedness 1	2150 µl 14000 µl 600 µl 1000 µl	**P** **C** **A** **NAD1**			Use only dry and freshly prepared. pH = 8.40
IV0	Inside out vesicles with 0 detergent	55µl 55 µl	**IOV** **F0**			
IVX	Inside out vesicles with Triton X-100	55 µl 55 µl	**IOV** **FX**			

7.3. Sodium-free buffers and solutions:

Name	Description	Amounts	components	Water is added until final vol.(ml)	Final molarity of main components	Notes (store at 4ºC if not otherwise noted)
KOH	KOH	28.06 g	KOH	100	KOH 5000	Store in tightly closed plastic bottle, as KOH will etch glass.
KK	KCl	29.82 g	KCl	100	KCl 4000	Must be stored in room temperature, not in cooling room, as solution is near saturation point**.**
TS	Tris, concentrated, acidic	39.41 g	Tris•HCl	250	Tris•HCl 1000	Store in cooling room.
TK	Tris concentrated.	30.29 g	Tris	250	Tris 1000	Store in cooling room
KFS	Potassium phosphate, acidic	13.609g	KH_2PO_4	100	Phosphate 1000	
KFB	Potassium phosphate,basic	22.823g	$K_2HPO_4•3H_2O$	100	Phosphate 1000	
KPBS	PBS where K^+ is cation.	1471 µl 8102 µl 35000 µl	**KFS** **KFB** **KK**	1000	Phosphate 9.57 K^+ 157.7 Cl^- 140	
EGN	EGTA, neutral	6.168 g 8000 µl	EGTA **KOH**	100	K_2-EGTA 200	Store in cooling room
PA	Prep-buffer A	2880 µl 2120 µl 28260 µl	**TS** **TK** **KK**	1000	Tris 5 K^+ 113 Cl^- 116	pH = 8,1
PB	Prep-buffer B	2640 µl 2360 µl 2500 µl 750 µl	**TS** **TK** **EGN** **KK**	1000	Tris 5 EGTA 0.5 K^+ 4 Cl^- 5.6	pH = 8.1
PC	Prep-buffer C	202 µl 298 µl	**TS** **TK**	1000	Tris 0.5 Cl^- 0.2	pH = 8.2
PHIS	Prep-buffer with 27.6% Histodenz	264 µl 236 µl 75 µl 150 µl 27.6 g	**TS** **TK** **KK** **EGN** Histodenz	100	Tris 5 K^+ 3.6 Cl^- 5.6 EGTA 0.3 Histodenz 336	pH = 8.1 Density = 1.146g/ml
PE	Prep- buffer E	292 µl 208 µl 150 µl 4300 µl	**TS** **TK** **EGN** **KK**	100	Tris 5 K^+ 173 Cl^- 175 EGTA 0.3	pH = 8.1 Density = 1.00g/ml

GT1	Density gradient #1	667µl 1333 µl	**PHIS** **PE**		Tris 5 K^+ 117 Cl^- 119 EGTA 0.3 Histodenz 112	pH = 8.1 Density = 1.048g/ml
GT2	Density gradient #2	4000 µl 2000 µl	**PHIS** **PE**		Tris 5 K^+ 60 Cl^- 62 EGTA 0.3 Histodenz 224	pH = 8.1 Density = 1.097g/ml
GT3	Density gradient #3	1500 µl	**PHIS**		Tris 5 K^+ 3.6 Cl^- 5.6 EGTA 0.3 Histodenz 336	pH = 8.1 Density = 1.146g/ml
MG	$MgCl_2$	5.083 g	$MgCl_2 \bullet 6H_2O$	25	$MgCl_2$ 1000	
MGF	$MgCl_2$ diluted	420 µl 980 µl	**MG** Water		$MgCl_2$ 300	
ATP	ATP-magnisum salt	1.522 g	Mg-ATP	10	Mg-ATP 300	Unstable, to be kept frozen or on ice for a minimum time.
CG	cGMP	0.00863 g 5000 µl	cGMP Water			

7.4. Solutions for ATPase assay for IOV:

Name	Description	Amounts	components	Water is added until final vol.(ml)	Final molarity of main components	Notes (store at 4ºC if not otherwise noted)
KOH	KOH	28.06 g	KOH	100	KOH 5000	Store in tightly closed plastic bottle, as KOH will etch glass.
KK	KCl	29.82 g	KCl	100	KCl 4000	Must be stored in room temperature, not in cooling room, as solution is near saturation point.
TS	Tris, concentrated, acidic	39.41 g	Tris•HCl	250	Tris•HCl 1000	Store in cooling room.
TK	Tris concentrated.	30.29 g	Tris	250	Tris 1000	Store in cooling room
EGN	EGTA, neutral	6.168 g 8000 µl	EGTA **KOH**	100	K_2-EGTA 200	Store in cooling room
ATP	ATP-magnisumsalt	1.522 g	Mg-ATP	10	Mg-ATP 300	Unstable, to be kept frozen or on ice for a minimum time.
SDS	Sodium dodecyl sulphate	6.00 g	Sodium dodecyl sulphate	50	Sodium dodecyl sulphate 12%	
OUA	Ouabain	0.29235 g	g-strophanthin $C_{29}H_{44}O_{12}$	5	Ouabain 100%	**Very Poisonous!!** Store in freezer. See important note 1 at bottom.
RB	Reagent B	3.00 g 7.395 g	Ascorbic acid HCl 37 %	50	Ascorbat 6% HCl 1500	**Dangerous!!** See important note 2 at bottom.
RC	Reagent C	1.00 g	Ammonium molybdate	100	Ammonium-molybdate 1 %	See important note 3 on bottom.
BC	Reagent BC	15000 µl 15000 µl	**RB** **RC**		Ascorbate 3% HCl 700 Ammonium molybdate 0.5%	See important note 4 on bottom.
CG	cGMP	0.00863 g 5000 µl	cGMP Water		cGMP 5	Store in freezer.
CGD	cGMP, diluted	600 µl 9400 µl	cGMP Water		cGMP 0.300	Store in freezer.
CG3	cGMP, diluted	3000 µl 7000 µl	cGMP Water		cGMP 1.50	Store in freezer.

IQ0	Incubation solution # 0 (basis)	1.2 µl 85.7 µl 73.5 µl 98 µl 147 µl 510 µl 1865 µl	**TS** **TK** **EGN** **ATP** **OUA** **KK** Water		Tris 51.72 EGTA 5.175 ATP 10.35 Mg^{2+} 10.35 Ouabain 5.175 K^{+} 728.6 cl^{-} 39.8	Total volume = 2840 µl Will be sufficient for 56 incubations when made into **IQ1** and **IQ2.** Prepare just before assay.
IQ1	Incubation solution # 1 (with cGMP)	1400 µl 48.3 µl	**IQ0** **CG3**		Tris 50.0 EGTA 5.00 ATP 10.0 Mg^{2+} 10.0 Ouabain 5.00 K^{+} 704.3 cl^{-} 715.1 cGMP 0.050	Total volume = 1448 µl Will be sufficient for 28 incubations. Prepare just before assay.
IQ2	Incubation solution # 2 (without cGMP)	1400 µl 48.3 µl	**IQ0** Water		Tris 50.0 EGTA 5.00 ATP 10.0 Mg^{2+} 10.0 Ouabain 5.00 K^{+} 704.3 cl^{-} 715.1 cGMP 0.0	Total volume = 1448 µl Will be sufficient for 28 incubations. Prepare just before assay.
FIQ1	Final incubate # 1	50 µl 150 µl 50 µl	**IQ1** Inhibitor solution or phosphate standard solution. vesicles		Tris 10.0 EGTA 1.00 ATP 2.0 Mg^{2+} 2.0 Ouabain 1.00 K^{+} 140.9 cl^{-} 143.0 cGMP 0.010	pH = 8.0 Iso-osmotic Gives ATPase activity with cGMP.
FIQ2	Final incubate # 2	50 µl 150 µl 50 µl	**IQ2** Inhibitor solution or phosphate standard solution. vesicles		Tris 10.0 EGTA 1.00 ATP 2.0 Mg^{2+} 2.0 Ouabain 1.00 K^{+} 140.9 cl^{-} 143.0 cGMP 0.0	pH = 8.0 Iso-osmotic Gives ATPase activity without cGMP.

Safety aspects:

Note1:

Wear protective gloves and a mask to prevent inhaling of dust when weighing Ouabain.

Note2:

HCl is very volatile and corrosive, and will burn skin. Use gloves. Don't inhale vapor. Use small volumes when weighing and keep lids on all containers.

Note3:

RC must be allowed to stand and equilibrate for at least two hours before being mixed with RB to form the color developing BC reagent. This is important, since the dissolved ammonium molybdate requires time to reach the equilibrium point that exists between the various molybdate complexes that characterize the solution. Failure in allowing sufficient time for equilibrium to occur has led to inconsistencies in the results obtained thereafter.

Note 4:

The stability and purity of this color reagent can then be tested spectrophotometricaly. It should give a reading of 0.052 units of optical density when measured at 680nm or 0.057-0.058 at 820nm. These values should remain constant over many hours. If they begin changing prior to the assay, then the reagents should be considered suspect and prepare a new.

7.5. Detailed Procedure of individual experiment

7.5.1. Preparation of IOV from human erythrocytes

This procedure must be done at 0 - 4°C, preferably in a cooling room, and yields a suspension of sealed IOV (and sealed ROV) void of any free phosphate. Tubes must always be carried on ice when transported between rooms.

01. Blood is withdrawn into EDTA-tubes. 20 ml is equivalent to one gradient tube later in this procedure, at step 19.
02. Centrifuge blood at 1000 g (2200 RPM) in original tubes for 15 minutes with max acceleration and max breaks.
03. Plasma and buffy coat are carefully removed with a disposable pasteur pipette and wasted.
04. Resuspend each 10 ml of packed erythrocytes in solution PA in a 50 ml centrifuge tube with round bottom. Do not fill the tube above the engraved line (ca 36 ml) on the tube.
05. Centrifuge tubes in a Beckman centrifuge at 1000 g (2875 RPM with rotor JA 25.50) for 10 minutes with max acceleration and slow breaks.
06. Supernatant is carefully removed and wasted. Also remove any remnants of a buffy coat. Repeat steps 4-6 two times more.
07. Lyse cells by quickly adding 10 volumes of ice cold solution PB to the erythrocytes. Transfer the lysate to a flask. Mix and resuspend the suspension vigorously. Use a disposable pasteur pipette to detach and resuspend remnants that still adheres to the tube wall/bottom. Pour lysate into tubes suited for centrifuge rotor JA-18. Make sure that the gasket of the tubes is mounted and the lid is tightly screwed on.
08. Centrifuge tubes in a Beckman centrifuge at 20000 g (11600 RPM with rotor JA-18) for 20 minutes with max acceleration and with breaks off. Because of the lack of breaks it will take ca 60-75 minutes (depending on number of tubes) before the centrifuge comes to a complete halt. When placing the tubes in a container filled with ice for transport between rooms, position the tube in such a way that it is impossible for the fluffy ghost sediment at the bottom of the tube to slide in any direction. This is a general rule that should be followed throughout this procedure.

09. Supernatant is carefully removed and wasted with an electrical high volume capacity pipette. Be careful not to touch any of the fluffy ghost (RBC membranes) sediment at the bottom of the tube. Switch to a disposable pasteur pipette for the delicate work of removing the last remnants of the supernatant.
10. After the first centrifugation post lysis, pour the ghost suspension to another clean centrifugation tube without removing the small hard yellowish pellet that is seen on the lower tube wall. The pellet is believed to contain contaminating proteases. After pouring, put tube in an upright position and wait a minute for the remaining viscous ghost suspension to slide down from the walls to gather at the bottom of the tube and then collect it carefully with a disposable pasteur pipette. Do not disturb the pellet. If traces of a yellowish pellet are seen in subsequent centrifugation steps, transfer ghost suspension to another clean tube as described.
11. Resuspend the ghosts in solution PB.
12. Centrifuge tubes in a Beckman centrifuge at 20000 g (11600 RPM with rotor JA-18) for 15 minutes with max acceleration and with slow breaks. Total centrifugation time is ca 25 minutes.
13. Be careful to remove all supernatant.
14. Repeat step 11-13 until the ghosts are milky white. It is important that all remnants of the supernatant consisting of solution PB is removed before proceeding to step 15, as a high ion concentration of ions surrounding the ghosts has a negative influence on the yield of sealed inside-out vesicles.
15. Measure the total volume of ghost suspension with a large pipette. Mix the ghosts with 50 volumes of solution PC and incubate the suspension for at least 2 hours to favor vesiculation.
16. Distribute the suspension in tubes suited for centrifuge rotor JA-18. The tubes must be of pair-wise equal weight to an accuracy of 2/100 of a gram for centrifugation balance in the next step. This is a general rule throughout the rest of this procedure for tubes that are to be centrifuged at high speeds.
17. Place tube pairs of equal weight diametrically opposed to each other in a Beckman centrifuge and centrifuge them at 47900 g (18000 RPM with rotor JA-18) for 30 minutes with max acceleration and with slow breaks.
18. Remove and waste supernatant.

19. Collect the membrane pellet with a disposable pasteur pipette by resuspending the pellet of the second tube in the pellet suspension collected from the first tube, and so on. Remnants are collected by washing the tube bottoms with a small amount of solution PC.
20. Homogenize the membrane pellet by forcing the suspension slowly both up and down through a 27” gauge cannula with the aid of a 1 ml syringe. Repeat this 5 times. If a very large volume of suspension is to be homogenized, use several syringes in parallel, bundled together with some tape.
21. The homogenate is applied on top of a Histodenz gradient tube (ca 2 ml for each tube). The tubes must be of pair wise equal weight to an accuracy of 2/100 of a gram for centrifugation balance in the next step.
22. Centrifuge tubes in a Beckman ultra centrifuge at 100 000 g (23700 RPM with rotor SW40) for at least 1 hour, or conveniently over night, with slow acceleration and breaks off. Be careful to balance diametrically opposed tubes.
23. Puncture the tube with a cannula and remove the upper band containing sealed IOV and sealed ROV with aid of a syringe. The lower band contains unsealed vesicles / ghosts of no interest. The tubes can be discarded.
24. Remove most of the Histodenz by resuspending the IOV in 100 volumes of solution PC, and centrifuge tubes in a Beckman centrifuge at 47900 g (18000 RPM with rotor JA-18) for 30 minutes with max acceleration and slow breaks.
25. Remove supernatant and collect pellet. Remnants of IOV are collected by washing the tube bottoms with a small amount of solution PC.
26. Switch on spectrophotometer in time and do sidedness test on IOV.

Sidedness Test of IOV of hRBC:

The enzyme acetylcholinesterase (AChE) is anchored to the cell membrane and is located at the intra- and the extracellular side of the membrane. For the inside out vesicles the situation is the opposite. By measuring the activity of this enzyme at the extravesicular side, the relative size of the IOV- and ROV-fractions of the vesicles can be found.

Example: IOV should give low AChE-activity, while ROV should give high AChE-activity.

The detergent Triton-X100 will make the membrane leaky. In the presence of this detergent, any extravesicular substrate will also become available for intravesicular enzyme; then both

IOV and ROV will give high AChE-activity. Therefore, if vesicles of unknown sidedness show low AChE-activity without detergent, but show high GAPD-activity in the presence of detergent, this would indicate that the vesicles are IOV.

1. You must need the solutions IS2, IV0, IVX, and ATCC.
   IS2 must be at room temperature while the other solutions are incubated on ice > 1 min before use.
2. In to a 1ml quartz cuvett, mix well in this order: 920 µl IS2 + 20µl IV0 + 63 µl ATCC (starts reaction), set reference to zero absorbance at 412 nm.
3. The increase in absorbance in 1 and 3 minutes is measured.
4. Repeat step 2 - 3 for a total of three parallels.
5. Do step 2 - 4 for IVX instead of IV0.

7.5.2. Phosphate assay and different phosphate concentrations

Different concentrations of phosphates (5 µM,15 µM, 25 µM, 50 µM, 100 µM and 250 µM) were prepared and in the presence of cGMP are incubated for suggested time in a solution with pH 8.0 at 37˚C that optimizes transport activity for the assay. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphates.

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ1 and IQ2 and keep them in water bath at 37˚C
3. Make FIQ1 by mixing 50µl IQ1 with 150 µl phosphate solution of known concentrations, and finally 50µl water. Total volume is 250 µl
4. Make FIQ2 by mixing 50µl IQ2 with 150 µl phosphate solution of known concentrations, and finally 50µl water. 250 µl
5. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a waterbath.
6. Stop reaction by adding 150 µl SDS. Be careful to not froth up the solution when adding the detergent.
7. Cool the tubes to room temperature for 5 minutes.
8. Start the development of color by adding 600µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
9. Pour solution into a cuvette and measure optical absorbance (OD) at 40 minutes and wavelength 680nm.

7.5.3. Phosphate assay and the effect of different concentrations of cGMP

Different concentrations of cGMP (0.5 µM, 5 µM, 25 µM, 50 µM and 100 µM) and Same concentrations of phosphates (0.5 µM, 5 µM, 25 µM, 50 µM and100 µM) were prepared and, in the absence of cGMP are incubated for suggested time in a solution with pH 8.0 at 37˚C that optimizes transport activity for the assay. Volume of cGMP was replaced with water. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphates.

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ1 and IQ2 and keep them in water bath at 37˚C
3. Make different concentrations of cGMP (0.5 µM, 5 µM, 25 µM, 50 µM and 100 µM).
4. Make different concentrations of phosphate solution (0.5 µM, 5 µM, 25 µM, 50 µM and 100 µM).
5. Make FIQ1 by mixing 50µl IQ1 with 150 µl water, and finally 50 µl phosphate solution of known concentration. Final volume is 250 µl.
6. Make FIQ2 by mixing 50µl IQ1 with 150 µl water, and finally 50 µl of different concentrations of cGMP. Final volume is 250 µl.
7. Repeat the step 5 and 6 for each concentration of Phosphate solution and cGMP solution.
8. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a water bath.
9. Stop reaction by adding 150 µl SDS. Be careful to not froth up the solution when adding the detergent.
10. Cool tubes to room temperature for 5 minutes.
11. Start the development of color by adding 600 µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
12. Pour solution into a cuvette and measure optical absorbance (OD) at 40 minutes.

7.5.4. Phosphate assay of IOV in presence and absence of cGMP

Inside out vesicles of hRBCs were stored at -70 C in microcentrifuge tubes. Required numbers of tubes was taken out of freezer and were kept in ice box at 4˚C. The thawn vesicle suspension was used to prepare analytes. These analytes of IOV suspension were added in the presence of cGMP to a solution with pH 8.0 at 37˚C that optimizes transport activity are incubated for suggested time. The transport reaction starts when IOV are mixed in. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphate

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ1 and IQ2 and keep them in water bath at 37˚C
3. Thaw IOVs and keep them in ice box at 4˚C
4. Make FIQ1 by mixing 50 µl IQ1 with 150 µl water, and finally 50 µl vesicle suspensions. Final volume is 250µl.
5. Make FIQ2 by mixing 50 µl IQ2 with 150 µl water, and finally 50 µl vesicles suspension. Final volume is 250µl.
6. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a water bath.
7. Stop reaction by adding 150 µl SDS. Be careful to not froth up the solution when adding the detergent.
8. Cool tubes to room temperature for 5 minutes.
9. Start the development of color by adding 600 µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
10. Pour solution into a cuvette and measure optical absorbance (OD) at 40 minutes.

7.5.5. Effect of different IOV concentrations on ATPase assay

Inside out vesicles of hRBCs were stored at -70 C in microcentrifuge tubes. Required numbers of tubes was taken out of freezer and were kept in ice box at 4˚C. The thawn vesicle suspension was used to prepare different dilutions of IOV (25%, 50%, 75%, and 100%). These concentrations of IOV suspension were added in the presence of cGMP in a solution with pH 8.0 at 37˚C that optimizes transport activity are incubated for suggested time for the assay. The transport reaction starts when IOV are mixed in. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphate.

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ1 and IQ2 and keep them in water bath at 37˚C
3. Thaw IOVs and keep them in ice box at 4˚C.
4. Prepare different dilution concentrations of IOV suspension (Vesicles Dilution Series, 25%, 50%, 75%, and 100%).
5. Make FIQ1 by mixing 50µl IQ1 with 150 µl water, and finally 50 µl vesicle suspension of required concentration. Final volume is 250 µl.
6. Make FIQ2 by mixing 50 µl IQ2 with 150 µl water, and finally 50 µl vesicles suspension of required concentration. Final volume is 250 µl.
7. Repeat the step 5 and 6 for all different dilution concentrations of IOV suspension.
8. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a water bath.
9. Stop reaction by adding 150 µl SDS. Be careful to not froth up the solution when adding the detergent.
10. Cool tubes to room temperature for 5 minutes.
11. Start the development of color by adding 600 µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
12. Pour solution into a cuvette and measure optical absorbance (OD) at 40 minutes.

7.5.6. Difference in absorbance in presence and absence of IOV

Inside out vesicles of human erythrocytes were stored at -70 C in microcentrifuge tubes. Required numbers of tubes was taken out of freezer and were kept in ice box at 4˚C. The thawn IOV were added in the absence of cGMP in a solution with pH 8.0 at 37˚C that optimizes transport activity are incubated for suggested time for the assay. The transport reaction starts when IOV are mixed in. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphates.

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ2 and keep it in water bath at 37˚C
3. Thaw IOVs and keep them in ice box at 4˚C.
4. Make FIQ1 by mixing 50 µl IQ2 with 150 µl water, and finally 50 µl vesicle suspensions. Final volume is 250 µl.
5. Make FIQ2 by mixing 50 µl IQ2 with 200 µl water. Final volume is 250 µl.
6. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a water bath.
7. Stop reaction by adding 150 µl SDS. Be careful to not froth up the solution when adding the detergent.
8. Cool tubes to room temperature for 5 minutes.
9. Start the development of color by adding 600 µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
10. Pour solution into a cuvette and measure optical absorbance (OD) at 40 minutes.

7.5.7. ATPase assay with higher IOV concentrations

Inside out vesicles of hRBC were stored at -70 C in microcentrifuge tubes. Six tubes were taken out of freezer and were kept in ice box at 4˚C. The thawn vesicle suspension was six times concentrated by using centrifuge. This concentrated suspension was used to prepare different dilutions of IOV by adding water (25%, 50%, 75%, and 100%). These concentrations of IOV suspension were added in the presence of cGMP in a solution with pH 8.0 at 37˚C that optimizes transport activity are incubated for suggested time for the assay. The transport reaction starts when IOV are mixed in. Reaction was stopped by using SDS 12%. Addition of coloring reagent made a color complex with free phosphates. Absorbance of analytes measured was directly proportional to the amount of available inorganic phosphates.

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ1 and IQ2 and keep them in water bath at 37˚C
3. Take 6 ml IOV suspension, thaw them and concentrate it to 1ml by centrifugation. Keep it in ice box at 4˚C.
4. Prepare different dilution concentrations from this 6 times concentrated IOV suspension (Vesicles Dilution Series, 25%, 50%, 75%, and 100%).
5. Make FIQ1 by mixing 50 µl IQ1 with 150µl water, and finally 50 µl vesicle suspension of required concentration. Final volume is 250 µl.
6. Make FIQ2 by mixing 50 µl IQ2 with 150 µl water, and finally 50 µl vesicles suspension of required concentration. Final volume is 250 µl.
7. Repeat the step 5 and 6 for each concentration of IOV suspension.
8. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a water bath.
9. Stop reaction by adding 150 µl SDS. Be careful to not froth up the solution when adding the detergent.
10. Cool tubes to room temperature for 5 minutes.
11. Start the development of color by adding 600 µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
12. Pour solution into a cuvette and measure optical absorbance (OD) at 40 minutes.

7.5.8. ATPase assay with higher IOV concentrations with modified protocol

Six microcentrifuge tubes of IOV stored at -70˚C were thawn and kept in ice box at 4˚C. Concentrated the thawn vesicle suspension six times, by using centrifuge. From this concentrated IOV stock suspension further dilutions of (25%, 50%, 75%, and 100%) were made and incubated in a solution of pH 8.0, with and without cGMP at 37˚C for 60 minutes that optimizes transport activity for the assay. The transport reaction starts when IOV are mixed in. Reaction was stopped and phospholipids were removed before incubation for color reaction. Color complex formation between coloring reagent and phosphates released gave absorbance which is directly proportional to the amount of available inorganic phosphates.

Procedure:

1. Use microcentrifuge tubes with snap cap.
2. Make IQ1 and IQ2 and keep them in water bath at 37˚C
3. Take 6 ml IOV suspension, thaw them and concentrate it to 1ml by centrifugation. Keep it in ice box at 4˚C.
4. Prepare different dilution concentrations from this 6 times concentrated IOV suspension (Vesicles Dilution Series, 25%, 50%, 75%, and 100%).
5. Make FIQ1 by mixing 50 µl IQ1 with 150 µl water, and finally 50 µl vesicle suspension of required concentration. Final volume is 250 µl.
6. Make FIQ2 by mixing 50 µl IQ2 with 150 µl water, and finally 50 µl vesicles suspension of required concentration. Final volume is 250 µl.
7. Repeat the step 5 and 6 for each concentration of IOV suspension.
8. Incubate FIQ1 and FIQ2 for 60 minutes at 37˚C in a water bath.
9. Stop reaction by chilling in ice cold water for 10 minutes.
10. Centrifuge at 13000 RPM for 15 minutes. Remove the supernatant by not disturbing the vesicles pellets.
11. Add 150 µl SDS. Be careful to not froth up the solution when adding the detergent and cool tubes to room temperature for 5 minutes.
12. Start the development of color by adding 600 µl BC and mix well without making bubbles as the clock is started immediately. Final volume is 1ml.
13. Pour solution into a cuvette and measure optical absorbance (OD) at 60 minutes.

7.5.9. ATPase assay of IOV with commercial kit

The ATPase colorimetric assay kit employs all reagents necessary for measuring ATPase activity. The kit contains P_iColorLock™ Gold reagent (an improved malachite green formulation) with additives to prevent background signals arising out of non-enzymatic ATP hydrolysis. Assay can be read anywhere in the wavelength range 590- 660nm.

Materials:

Components in the ATP assay kit (Store at 4℃):

- 1 X 10ml of P_iColorLock™ Gold* (1 X 25ml)
- 1 X 0.25ml of Accelerator (1 X 0.5ml)
- 1 X 5ml of stabilizer (1 X 10ml)
- 1 X 1.5ml of 0.1M $MgCl_2$ (2 X 1.5ml)
- 1 X 5ml of 0.5 M Tris pH 7.5 (1 X 10ml)
- 1 X 5ml of 0.1mM P_i standard (1 X 10 ml)

Components in the ATP assay kit (Store at -70°C):

- 2 X 1ml specially purified ATP (5 X1ml)

Other components:

- Inside out vesicles.
- Water
- 10µM cGMP.

*Exercise caution – this reagent contains 5 M HCl. Wear gloves and safety glasses.

Procedure:

1. Make without cGMP substrate / buffer mix. The assay kit is supplied with 0.5 M Tris assay buffer pH 7.4. Most ATPase will require a metal ion cofactor. $MgCl_2$ (0.1M) is supplied with the kit. Table 1 (below) shows the volumes required to make up SB mix for selected no. of cuvettes. These volumes result in final buffer and Mg^{2+} concentrations (i.e. after your enzyme has been added) of 50mM and 2.5 mM, respectively. The final concentration of purified ATP is 0.5mM.

No. of cuvettes	0.5M Buffer (µl)	0.1M MgCl2 (µl)	10 mM ATP (µl)	Water (µl)
1	74.2	18.55	37.1	241.15
14	1038.8	259.7	519.4	3376.1

2. Make with cGMP substrate / buffer mix. The assay kit is supplied with 0.5 M Tris assay buffer pH 7.4. Most ATPase will require a metal ion cofactor. $MgCl_2$ (0.1M) is supplied with the kit. Other then assay kit components, cGMP (10µM) was also provided to make with cGMP substrate / buffer mix. Table 2 (below) shows the volumes required to make up SB mix for selected no. of cuvettes. These volumes result in final buffer and Mg^{2+} concentrations (i.e. after your enzyme has been added) of 50mM and 2.5 mM, respectively. The final concentration of purified ATP is 0.5mM.

No. of cuvettes	0.5M Buffer (µl)	0.1M MgCl2 (µl)	10 mM ATP (µl)	10µM cGMP	Water (µl)
1	74.2	18.55	37.1	4.9	236.6
14	1038.8	259.7	519.4	69.25	3306.7

3. Make 'Gold mix' by adding Accelerator to P_iColor Lock™Gold.

4. Prepare 'Gold mix shortly before the reagent is required by adding 1/100 volume of Accelerator to P_iColorLock™Gold (see table 3 below).

No. Of Cuvettes	P_i Color Lock™ Gold (µl)	Accelerator (µl)
1	185	1.85
14	2590	25.9

5. Set up assays with ATPase (not supplied); 371µl vesicles plus 371µl substrate/buffer mix with cGMP and without cGMP. By fixing the assay time 30 minutes, and temperature 25˚ C.

6. Stop reaction by chilling in ice cold water for 10 minutes.

7. Centrifuge at 13000 RPM for 15 minutes. Remove the supernatants.

8. Add 185µl of Gold mix to stop reactions.

9. After 2 minutes, add 74 µl of stabilizer.

10. After 30 minutes read the absorbance of cuvettes at a wavelength 635nm.

7.6. Results, raw data of individual experiment

7.6.1. Results for sidedness test of IOV

Three different batches of IOV were prepared and tested for sidedness, following yields were obtained.

Table a. for batch 1:

IV0	Mean IV0	IVX	Mean IVX
0.86	0.93	1.80	1.86
0.95		1.86	
0.99		1.92	

Table a contains mean value for IV0 (0.93) calculated by three times measured optical absorbance (OD) at 40 minutes and the mean value for IVX (1.86) calculated by three times measured optical absorbance (OD) at 40 minutes.

Calculation of percentage of inside out vesicles

Percentage of right side out vesicles = $\frac{IV0}{IVX}$

$= \frac{0.93}{1.86}$

$= 0.5 \sim 50\%$

Percentage of inside out vesicles = 100 – ROV % age

= 50 %

After experiment 1 yield of Inside out vesicles was 50%. Total obtained volume of vesicles were distributed in 1mL aliquots, labeled them with percentage, and stored at -70℃ to reuse them as per required.

Table b. for batch 2:

IV0	Mean IV0	IVX	Mean IVX
0.890	0.892	1.191	1.195
0.896		1.207	
0.889		1.189	

Table b contains mean value for IV0 (0.892) calculated by three times measured optical absorbance (OD) at 40 minutes and the mean value for IVX (1.195) calculated by three times measured optical absorbance (OD) at 40 minutes.

Calculation of percentage of IOV

Percentage of right side out vesicles = $\frac{IV0}{IVX}$

$$= \frac{0.892}{1.195}$$

$$= 0.74 \sim 74\%$$

Percentage of inside out vesicles = 100 – ROV % age

= 26 %

After experiment 2, yield of IOV was 26%. Total obtained volume of vesicles were distributed in 1mL aliquots, labeled them with percentage and stored at -70℃ to reuse them as per required.

Table c. for batch 3:

IV0	Mean IV0	IVX	Mean IVX
0.889	0.893	1.217	1.235
0.896		1.207	
0.894		1.220	

Table c contains mean value for IV0 (0.893) calculated by three times measured optical absorbance (OD) at 40 minutes and the mean value for IVX (1.235) calculated by three times measured optical absorbance (OD) at 40 minutes.

Calculation of Percentage of IOV:

Percentage of right side out vesicles = $\frac{IV0}{IVX}$

$= \frac{0.893}{1.235}$

$= 0.72 \sim 72\%$

Percentage of inside out vesicles = 100 – ROV % age

= 28 %

After experiment 3, yield of IOV was 28%. Total obtained volume of vesicles were distributed in 1mL aliquots, labeled them with percentage and stored at -70℃ to reuse them as per required.

7.6.2. Effect of different IOV concentrations on ATPase assay:

To verify the results, this experiment was performed three times according the same protocol and following results were obtained.

Table d:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	Mean	SD	a	b	c	Mean	SD	%	SD
25	0,08	0,08	0,082	0,081	0,0009	0,036	0,011	0,015	0,021	0,0110	58,82	0,011005
50	0,082	0,08	0,096	0,086	0,0071	0,018	0,025	0,03	0,024	0,0049	60,78	0,008654
75	0,096	0,09	0,094	0,093	0,0025	0,03	0,027	0,02	0,026	0,0042	65,68	0,004876
100	0,104	0,103	0,099	0,102	0,0022	0,037	0,031	0,033	0,034	0,0025	66,66	0,0033

Table e:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	Mean	SD	a	b	c	Mean	SD	%	SD
25	0,039	0,031	0,039	0,036	0,0038	0,033	0,027	0,022	0,027	0,0045	6,52	0,005869
50	0,058	0,061	0,065	0,061	0,0029	0,051	0,055	0,04	0,049	0,0063	8,69	0,00696
75	0,089	0,089	0,07	0,083	0,0090	0,11	0,115	0,101	0,109	0,0058	-18.11	0,010667
100	0,141	0,14	0,132	0,138	0,0040	0,114	0,12	0,125	0,120	0,0045	13.04	0,006037

Table f:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	Mean	SD	a	b	c	Mean	SD	%	SD
25	0,034	0,037	0,036	0,036	0,036	0,036	0,029	0,029	0,031	0,0033	9,70	0,03637
50	0,055	0,063	0,061	0,060	0,060	0,060	0,043	0,043	0,049	0,0082	11,65	0,061772
75	0,07	0,086	0,077	0,078	0,078	0,078	0,061	0,061	0,067	0,0085	13,59	0,080667
100	0,1	0,099	0,103	0,101	0,101	0,101	0,087	0,087	0,092	0,0065	14,56	0,101098

Table (d, e and f) explains about absorbance (at 680nm) of different concentrations of IOV with and without cGMP, also the Percentage of difference of absorbance and standard deviations.

To measure ATPase activity, for each IOV concentration e.g. (25%) three parallels with cGMP and three parallels for same IOV concentration but without cGMP were prepared to avoid possible errors. Absorbance measured at wavelength 680 nm for each parallel is presented by a, b, and c also calculated the mean and standard deviation for both (IOV concentration with and without cGMP).Percentage of difference of absorbance was calculated from ((Mean with cGMP – Mean without cGMP) / Max. absorbance) x 100

7.6.3. ATPase Assay with higher IOV concentrations

To verify the results, this experiment was performed three times according the same protocol and following results were obtained.

Table g:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	Mean	SD	a	b	c	Mean	SD	%	SD
25	0,023	0,019	0,013	0,018	0,0041	0,012	0,018	0,01	0,013	0,0034	5,31	0,005333
50	0,038	0,034	0,038	0,037	0,0019	0,037	0,034	0,037	0,036	0,0014	1,06	0,002357
75	0,068	0,072	0,059	0,066	0,0054	0,037	0,041	0,037	0,038	0,0019	29,78	0,005754
100	0,093	0,097	0,091	0,094	0,0025	0,058	0,058	0,047	0,054	0,0052	42.55	0,005754

Table h:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	Mean	SD	a	b	c	Mean	SD	%	SD
25	0,228	0,222	0,22	0,223	0,0034	0,149	0,145	0,147	0,147	0,0016	80,85	0,003771
50	0,242	0,249	0,24	0,244	0,0039	0,185	0,183	0,179	0,182	0,0025	65,95	0,004595
75	0,273	0,276	0,272	0,274	0,0017	0,205	0,202	0,2	0,202	0,0021	76,59	0,002667
100	0,301	0,3	0,299	0,300	0,0008	0,238	0,245	0,236	0,240	0,0039	63,82	0,003944

Table i:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	Mean	SD	a	b	c	Mean	SD	%	SD
25	0,015	0,015	0,014	0,015	0,015	0,012	0,013	0,016	0,014	0,0017	1,69	0,014654
50	0,031	0,03	0,027	0,029	0,029	0,027	0,022	0,015	0,021	0,0049	13,56	0,029196
75	0,046	0,049	0,034	0,043	0,042	0,038	0,038	0,042	0,039	0,0019	6,78	0,042042
100	0,059	0,062	0,057	0,059	0,059	0,052	0,054	0,056	0,054	0,0016	8,47	0,059467

Table (g, h and i) shows absorbance (at 680 nm) of different concentrations of 6 X concentrated IOV with and without cGMP, also the Percentage of difference of absorbance and standard deviations.

To measure ATPase activity, for each IOV concentration e.g. (25%) three parallels with cGMP and three parallels for same IOV concentration but without cGMP were prepared to avoid possible errors. Absorbance measured at wavelength 680 nm for each parallel is presented by a, b, and c also calculated the mean and standard deviation for both (IOV concentration with and without cGMP). Percentage of difference of absorbance was calculated from ((Mean with cGMP – Mean without cGMP) / Max. absorbance) x 100.

7.6.4. ATPase Assay with higher IOV concentrations with modified protocol

To verify the results, this experiment was performed three times according the same protocol and following results were obtained.

Table j:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	mean	SD	a	b	c	mean	SD	%	SD
25	0,08	0,08	0,082	0,081	0,0009	0,036	0,011	0,015	0,021	0,0110	58,82	0,011005
50	0,082	0,08	0,096	0,086	0,0071	0,018	0,025	0,03	0,024	0,0049	60,78	0,008654
75	0,096	0,09	0,094	0,093	0,0025	0,03	0,027	0,02	0,026	0,0042	65,68	0,004876
100	0,104	0,103	0,099	0,102	0,0022	0,037	0,031	0,033	0,034	0,0025	66,66	0,0033

Table k:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	mean	SD	a	b	c	mean	SD	%	SD
25	0,039	0,031	0,039	0,036	0,0038	0,033	0,027	0,022	0,027	0,0045	7,5	0,005869
50	0,058	0,061	0,065	0,061	0,0029	0,051	0,055	0,04	0,049	0,0063	10	0,00696
75	0,110	0,115	0,101	0,109	0,0058	0,089	0,089	0,08	0,086	0,0042	19,16	0,00718
100	0,114	0,120	0,125	0,120	0,0045	0,141	0,14	0,132	0,095	0,0576	20,83	0,057779

Table l:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	mean	SD	a	b	c	mean	SD	%	SD
25	0,034	0,037	0,036	0,036	0,036	0,027	0,029	0,023	0,026	0,0025	9,9	0,036308
50	0,055	0,063	0,061	0,060	0,061	0,046	0,043	0,048	0,046	0,0021	13,86	0,061257
75	0,07	0,086	0,077	0,078	0,080	0,064	0,061	0,064	0,063	0,0014	14,85	0,080235
100	0,1	0,099	0,103	0,101	0,101	0,085	0,087	0,083	0,085	0,0016	15,84	0,100902

Table (j, k and l) is about absorbance (at 680 nm) of 6 X concentrated IOV of different dilution concentrations with and without cGMP, also the difference of absorbance in percent and standard deviations with modified protocol for minimizing the presence of phospholipids.

To measure ATPase activity, for each IOV concentration e.g. (25%) three parallels with cGMP and three parallels for same IOV concentration but without cGMP were prepared to avoid possible errors. Absorbance measured at wavelength 680 nm for each parallel is represented by a, b, and c also calculated the mean and standard deviation for both (IOV concentration with and without cGMP). Percentage of difference of absorbance was calculated by ((Mean with cGMP – Mean without cGMP) / Max. absorbance) x 100.

7.6.4. ATPase assay of IOV with commercial kit

To verify the results, this experiment was performed three times according the same protocol and following results were obtained.

Table m:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	mean	SD	a	b	c	mean	SD	%	SD
25	0,2	0,23	0,223	0,218	0,0128	0,047	0,036	0,04	0,041	0,0045	0,177	0,013597
50	0,197	0,24	0,196	0,211	0,0205	0,004	0,029	0,067	0,033	0,0259	0,178	0,033039
75	0,339	0,34	0,363	0,347	0,0111	0,135	0,137	0,151	0,141	0,0071	0,206	0,013174
100	0,375	0,391	0,415	0,394	0,0164	0,099	0,12	0,148	0,122	0,0201	0,272	0,025944

Table n:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	mean	SD	a	b	c	mean	SD	%	SD
25	0,356	0,356	0,372	0,361	0,0075	0,349	0,377	0,326	0,351	0,0209	0,01	0,022176
50	0,778	0,75	0,681	0,736	0,0408	0,73	0,733	0,71	0,724	0,0102	0,012	0,042021
75	0,921	0,949	0,906	0,925	0,0178	0,875	0,877	0,881	0,878	0,0025	0,047	0,017994
100	1,395	1,456	1,373	1,408	0,0351	1,265	1,287	1,322	1,291	0,0235	0,117	0,042232

Table o:

IOV conc. %	Absorbance											
	With cGMP					Without cGMP					Difference of Absorbance in percent.	
	a	b	c	mean	SD	a	b	c	mean	SD	%	SD
25	0,252	0,29	0,273	0,272	0,0155	0,236	0,208	0,219	0,221	0,0115	0,051	0,019345
50	0,503	0,523	0,444	0,490	0,0335	0,437	0,437	0,44	0,438	0,0014	0,052	0,033566
75	0,706	0,642	0,648	0,665	0,0289	0,624	0,603	0,608	0,612	0,0090	0,053	0,030218
100	0,764	0,776	0,796	0,779	0,0132	0,72	0,72	0,716	0,719	0,0019	0,06	0,013333

Table (m, n and o) shows absorbance (at 635 nm) of 6 X concentrated IOV of different dilution concentrations with and without cGMP, also the difference of absorbance in percent and standard deviations with Innova Biosciences ATPase assay kit.

To measure ATPase activity, for each IOV concentration e.g. (25%) three parallels with cGMP and three parallels for same IOV concentration but without cGMP were prepared to avoid possible errors. Absorbance measured at wavelength 635 nm for each parallel is presented by a, b, and c also calculated the mean and standard deviation for both (IOV concentration with and without cGMP). Percentage of difference of absorbance was calculated by ((Mean with cGMP – Mean without cGMP) / Max. absorbance) x 100.